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# ERRATA AND AUTHORS' EMENDATIONS

Page 82, line 2, "figure 1,  $K$ " should be "figure 1,  $I$ ."

Page 84, line 29, "(fig. 1,  $C$ )" should be "(fig. 2,  $C$ )."

Page 106, line 11, "*pisi*" should be "*batalas*."

Page 154, line 13, "22 days" should be "26 days."

Page 162, twelfth line from bottom,  $e - \rho^x$  should be  $e^{-\rho^x}$ .

Page 166, fifth line from bottom,  $e - \sigma^t$  should be  $e^{-\sigma^t}$ .

Page 168, equation (12) should be

$$B_{sh} = Z_1 I \alpha / \rho (1 - e^{-\rho l'}) + Z_2 I \frac{\gamma}{\sigma} (1 - e^{-\sigma t}) e^{-\sigma d} e^{\rho l'} + Z_1 I \frac{\alpha}{\rho} (e^{-\rho l'} - e^{-\rho b}) e^{-\sigma t} \\ + I A f(\theta \psi) e^{-\rho l} e^{-\sigma t}.$$

Page 169, line 2,  $\Sigma$  should be  $\sigma$ .

Page 170, equation (21), the denominator should be  $Z_1 \frac{\alpha}{\rho} \frac{\mp V}{1 \pm V}$ .

Page 173, equation (25), the term carrying the  $A$  should be  $\frac{A f(\theta \psi)}{Z_1 \alpha / \rho} - 1$ .

Page 185, table 3, footnote 1, second line, "face of leaflet" should be "surface of leaflet."

Page 280, table 25, under heading "Analysis of Variance," last column, second line, ">99:1" should be ">19:1."

Page 327, figure 3, " $F$ " should be omitted from the legend.

Page 361, third and twelfth lines from bottom, "modulation" should be "modulation."

Page 395, eighth line from bottom, "*sativus*" should be "*sativa*."

Page 448, table 3, column 10, ".7" should be "-.7."

Page 497, table 2, "No. 78 Sagraín," the missing figures for columns 4 to 8, respectively, are 72.84; 23; 44; 50; 52."

Page 537, line 10, "nicotine acid" should be "nicotinic acid."

Page 546, footnote 2, "*phaseolica*" should be "*phaseolicola*."

Page 551, table 3, column 4, average for 1937, "131.05" should be "131.09."

Page 552, eighth line from bottom, "12 varieties" should be "11 varieties."

Page 605, line 13, "45-kr." should be "45-kv."

Page 609, second paragraph, line 4, "60°" should be "50°."

Page 619, under Review of Literature, line 2, "estrongenic" should be "estrogenic."

Page 708, table 14, column 3, line 10, "Room to 25" should be "Room to 35."



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## CARBOHYDRATE METABOLISM AND WINTER HARDINESS OF WHEAT<sup>1</sup>

By ERIC KNEEN, *assistant agricultural chemist and assistant plant pathologist*, and  
M. J. BLISH, *agricultural chemist, Nebraska Agricultural Experiment Station*

### INTRODUCTION

Research conducted by many workers during the past half century has resulted in a body of evidence which indicates an important connection between soluble carbohydrates and the cold resistance of winter-hardy cereals. On the other hand, the frequent appearance of confusing and contradictory results and conclusions justifies additional research. Frequently cold resistance has been confused with winter hardiness; i. e., the resistance to frost injury at a specific time during hardening has received more attention than the development and maintenance of cold resistance throughout the winter period. The exact nature of the hardening process and the role of various factors in this process are of fundamental interest.

In the breeding of desirable varieties of winter cereals the incorporation of winter hardiness is usually a necessary precaution. Rapid and reliable methods for varietal characterization which may supplement the field and controlled freezing tests being conducted for this purpose are greatly to be desired. There is, then, a need for information regarding the exact relationship between carbohydrate metabolism and winter hardiness and also regarding the possibility of adapting such information to the differentiation of varieties on the basis of their winter hardiness. In the past such studies have been largely confined to field-grown material subject to the effect of adverse environmental conditions. Unquestionably this lack of control accounts for many of the contradictions found in the literature. An examination of the relationship between carbohydrate metabolism and winter hardiness of wheat as determined under controlled conditions has been undertaken with the results and conclusions as herewith reported.

### LITERATURE REVIEW

No single source as yet provides an adequate review of the literature bearing on the relationship of soluble carbohydrates to winter hardiness of cereals. However, papers by Schaffnit (30),<sup>2</sup> Åkerman (1), Fuels (9), and Levitt and Scarth (17) include rather extensive

<sup>1</sup> Received for publication April 16, 1940. Contribution from the Departments of Agricultural Chemistry and Plant Pathology, Nebraska Agricultural Experiment Station. Journal Series paper No. 262 of the Nebraska Agricultural Experiment Station.

<sup>2</sup> Italic numbers in parentheses refer to Literature Cited, p. 24.



literature reviews, and the reader is referred to them for a more adequate treatment than space permits in the present paper.

The work of numerous investigators has established that coincident with a pronounced increase in cold resistance during the advent of the low temperatures of fall and winter, there is a pronounced increase of soluble carbohydrates in the wheat plant. A relationship has been established between low-temperature hardening, sugar accumulation, and radiation intensity. Among others, Tumanov (36, 37), Mudra (20), Dexter (5), and Constantinescu (4) have demonstrated that light is necessary for the normal hardening of winter cereals and have likewise shown that the lack of hardening under light deficiency conditions is accompanied by a lack of sugar accumulation. Further emphasizing the importance of light in the development of cold resistance, Tumanov (37) and Suneson and Peltier (34) showed that any shading of the hardening plants, whether continuous or periodic, resulted in decreased cold resistance.

Attempts to express varietal differences in winter hardiness of cereals by carbohydrate analysis have not been entirely successful. Akerman (1) was able to arrange 11 varieties of winter wheat in the order of their relative cold resistance by means of sugar and dry-matter determinations. Lebedineev et al. (16) experimented with 44 varieties of wheat, 16 of rye, and 4 of barley and showed that while individual variation in cold resistance was difficult to express by sugar analysis, the varieties could be arranged into hardy, medium-hardy, and nonhardy groups by this means. Mudra (20) was likewise able to establish a correlation between analysis and field-exhibited cold resistance. On the other hand, Newton (21, 22) reported that dry matter and sugar increases, while associated with hardening, could not be used for varietal differentiation. Martin (19) and Salmon and Fleming (27) found a lack of correlation between cell sap solids and cold resistance of winter wheat. Timofejeva (35) and Balde (2) reported that there was no clear-cut agreement between sugar content and varietal differences in cold resistance. Govorov (10) found no correlation between dry-matter content and hardiness and no difference between winter and spring cereals in this respect. Janssen (11), in an experiment designed to study the effect of date of planting on winter hardiness of wheat, found that the plants of one of the planting dates which resulted in low cold resistance, contained the highest content of sugars. Further, Dexter (8) reported that an increase in sugars at low temperatures may or may not be accompanied by decidedly increased hardiness.

It is obvious from the above that more information must be obtained before any comprehensive picture of the relationship between winter hardiness and carbohydrate metabolism may be drawn.

## MATERIALS AND METHODS

### EXPERIMENTAL MATERIAL

Winter wheat (*Triticum aestivum* L.) was selected as a cereal grain readily adaptable to experimental procedure and one of which samples covering a wide range of known varietal winter hardiness are easily procurable. Table 1 shows the winter-hardiness characteristics, as outlined by Quisenberry (25), for the six varieties used throughout the present study. It may be seen from table 1 that the actual winter

survival in the uniform winter-hardiness nurseries of the Great Plains area during the period 1930-37 ranged from 71.5 percent for Minhardi to 44.7 percent for Fulcaster. In addition to the above varieties of winter wheat, a spring wheat, either Marquis or Ceres, and Tennessee winter barley were used on occasion for comparative purposes.

The seedling material used for hardening, dehardening, and freezing experiments was obtained by growing the grain in flats of fertile greenhouse soil in the greenhouse for a 3-week period following the date of planting. An attempt was made to hold the greenhouse temperature at approximately 67° to 70° F., but in the case of the early fall planting outside conditions caused a notable rise in the average growing temperature. Each flat contained all six varieties of winter wheat and in each series of six flats six different positions were occupied by each variety.

TABLE 1.— *Winter-hardiness classification of the six varieties of winter wheat used for controlled studies*

Variety	C. I. <sup>1</sup> No	Description	Field survival 1930-37 <sup>2</sup>	
			Weighted average	In ratio to Karkof
			Percent	Percent
Minhardi	5149	Soft red winter.	71.5	123.3
Minturki	6155	Hard red winter	67.4	110.2
Cheyenne	8885	do	59.7	102.9
Kawvale	8180	Soft red winter	58.2	93.6
Blackhull	6251	Hard red winter.	45.8	79.0
Fulcaster	6471	Soft red winter	44.7	77.1

<sup>1</sup> C. I. denotes accession number of the Division of Cereal Crops and Diseases, Bureau of Plant Industry U. S. Department of Agriculture.

<sup>2</sup> Data for average field survival taken direct from published data of Quisenberry (25).

During the winter of 1937-38 three experiments were conducted, the same six varieties of winter wheat being used throughout. The material of experiment A was hardened through the period October 27 to December 10, 1937; experiment B through December 13, 1937, to February 8, 1938; and experiment C through February 10 to April 4, 1938. In this manner hardening was carried on under both decreasing and increasing day lengths. Experiments not reported in detail in this paper indicated that controlled hardening of the type used results in the maximum level of reserves being attained in about 50 days. For example, the average sucrose and dry-matter content for the crowns of two varieties of winter wheat were 15.99 and 30.22 percent, respectively, after 48 days of hardening as compared with 15.73 and 29.70 percent after 70 days. Accordingly, the experiments were terminated after about 7 to 8 weeks of hardening.

#### HARDENING, DEHARDENING, AND FREEZING

The control equipment for the experimental hardening and freezing of crop plants has been described by Peltier (24). In essentials the hardening room consists of a double-glassed greenhouse held by refrigeration at any constant temperature between freezing and normal growing temperatures. The freezing room may be held with little variation at any desired temperature from 0° to -30° C. Through-

out the work herein reported the hardening room temperature was maintained at approximately 2°.

The experimental plants were moved from the warm greenhouse to the hardening room at the 3-week seedling stage and held thereafter continuously at approximately 2° C., and were watered when necessary. As desired, samples were taken for analysis, flats were removed to the freezing chamber for survival tests, or were transferred back to the warm greenhouse for dehardening.

For purposes of this report the term "dehardening" means the process involved when a previously low-temperature hardened plant is caused to lose a degree of its cold resistance by the influence of the higher temperatures conducive to the transition from dormancy to active growth.

#### ANALYTICAL METHODS

##### SAMPLING

Analyses are reported for several somewhat different types of material. During the early part of the year (experiments A and B) samples referred to as "leaves" represented the section of the seedling above ground level, material other than leaves being only a small part of the whole. When both crown and leaf tissue were sampled, as in experiment C, the whole plant, after removal of the roots, was divided into two parts: (1) The leaf blades and (2) the remaining crown, stem, and leaf-sheath materials. This crown and stem tissue is referred to as "crown." In one instance—the dehardening phase of experiment B—the whole plant, after removal of the roots, was used for analysis.

No matter what plant section was selected, each sample represented a composite of from 30 to 50 plants, i. e., a composite containing from 5 to 8 plants from each of 6 flats. The selected material, after cleaning and removal of any dead tissue, was cut up finely with scissors, mixed thoroughly, and placed in an aluminum moisture can of appropriate size. All operations were conducted as rapidly and uniformly as possible. Sampling of hardened material was performed in the hardening room. Sampling of unhardened or dehardened material was done in the warm greenhouse but the sealed cans were placed in the hardening room as soon as obtained. Unless otherwise indicated, all sampling was conducted between 10 and 11 a. m.

Following the sampling, the material was kept under refrigeration except during the brief periods when it was being transported to the laboratory or being weighed. It was established that minor variations in this sampling procedure had essentially no influence on the subsequent dry matter and sugar determination.

##### DETERMINATION OF DRY MATTER

An appropriate amount (4 to 5 gm.) was rapidly weighed into a small aluminum moisture can and the cover tightly affixed. This can with its contents was placed on a ½-inch solid aluminum shelf in an oven held at 140° C. and left there for 15 minutes. By this means steam is produced almost immediately inside the can, thereby rapidly inactivating the enzymes without charring. Following this preheating treatment, the material was dried overnight under vacuum at 90°, cooled in a desiccator, reweighed, and the percent dry matter calcu-

lated. In addition, this method of heating and drying produces a dry sample which, if desired, may be quantitatively transferred to a flask and extracted for sugar determination.

#### DETERMINATION OF SUGARS

Either 4 to 5 gm. of the green material or the entire residue resulting from one of the dry-matter determinations was placed in a 100-cc. Kohlrausch volumetric flask. A few drops of toluol were added and the flask was covered with a watch glass and then gently heated to permit permeation of the plant material with toluol vapor. Fifty to sixty cubic centimeters of boiling water were then added and extraction carried out for 1 hour on the steam bath. Longer periods of extraction have not proved beneficial and under the above conditions neither has the addition of calcium carbonate for neutralization.

Following extraction, reducing sugars were determined by the ferricyanide method, as described by Blish and Sandstedt (3), using 0.10 N reagents as suggested by Sandstedt (28). To the cooled extract 4 cc. of 10-percent sulfuric acid and 4 cc. of 12-percent sodium tungstate solution were added, the volume brought up to 100 cc. with water, filtered, and a 5-cc. aliquot of the filtrate taken for reduction. It was realized that all of the nonsugar material which is capable of reducing ferricyanide under the conditions of the method is not removed by the clarifying agents used. A conventional copper-reduction method following alcohol extraction and lead acetate clarification was used as a standard and some 25 samples run by both this and the ferricyanide method. Assuming true values for reducing sugars by the copper-reduction method, it was possible to calculate the reducing-sugar content of the material from the volume of ferricyanide reduced. Reducing sugars are conveniently expressed as glucose.

Sucrose was determined by a slight modification of the method proposed by Sandstedt (29). A 5-cc. aliquot of the filtrate used above for reducing-sugar determination was placed in a test tube and heated in the boiling water bath for 7 minutes. This proved adequate to hydrolyze the sucrose. After cooling, the ferricyanide solution was added and reduction carried on as customary. Subtraction of the volume of ferricyanide reduced in the previous determination of reducing sugars from this final total volume of reduced ferricyanide gave the reduction attributable to inverted sucrose. The use of the sucrose-ferricyanide table of Sandstedt (29) permitted ready transposition into milligrams of sucrose. A comparison of this method with the conventional hydrolysis and copper-reduction procedure showed close agreement between the two.

#### METHOD OF RECORDING RESULTS

There has been considerable variation in the manner in which investigators have expressed the sugar content of plant material. Sugar content has been reported as percent of dry weight, percent of green weight, weight per 100 plants, and as percent in the water actually present in the fresh plant tissue. It is obvious that if the sugar content of the plant is considered significant by reason of its osmotic effect its role as a solute must be the important factor. Whether or not osmotic relationships are of prime significance in cold

resistance there is no apparent disadvantage in using this method of expression. Accordingly, sugar is recorded as the percent glucose, or sucrose, in the total water actually present in the fresh, green seedling material at the time of analysis.

## EXPERIMENTAL RESULTS

### THE HARDENING PROCESS

#### GENERAL CONSIDERATIONS

To be comparable to natural field hardening, any method of artificial low-temperature hardening should produce comparable plants

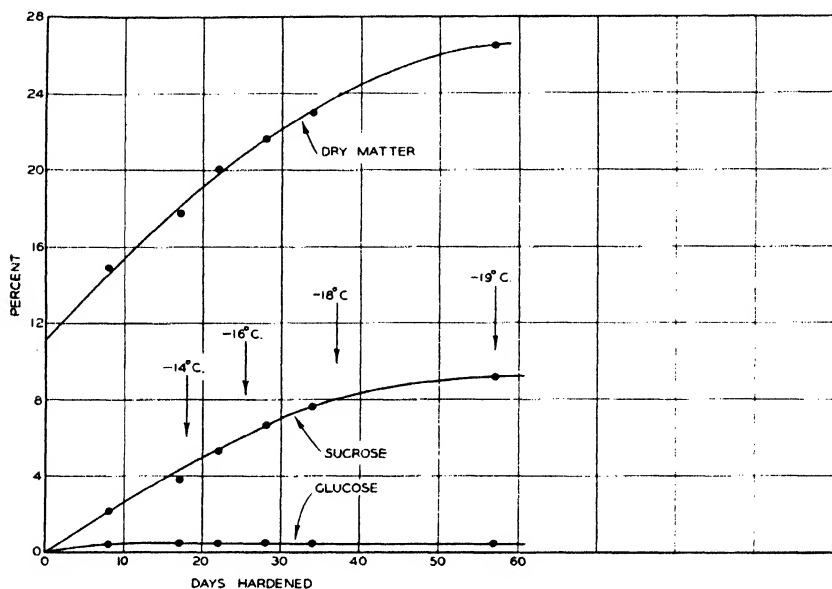


FIGURE 1.—Increase of dry matter and sugars in the leaves of Cheyenne wheat during the 8 weeks of controlled hardening. Inserted temperatures are those at which a 24-hour exposure resulted in 20 percent of the plants being killed.

both as regards physiological and morphological make-up. Suneson and Peltier (32) have indicated that with wheat seedlings under controlled low-temperature hardening ( $29^{\circ}$  to  $35^{\circ}$  F.) the maximum degree of cold resistance is reached in 3 weeks. They likewise found (34) that after this period of hardening the cold resistance of the plants, as measured by freezing tests, was much less than that of field-hardened material. Their data indicate effective freezing temperatures to be of the order of  $-11^{\circ}$  C. for the control-hardened as compared to  $-20^{\circ}$  to  $-24^{\circ}$  for the field-hardened material. Further suggesting that control and field-grown plants were not strictly comparable is the observation of these authors (32) that during 3 weeks of hardening the decumbent stature of the seedlings increased only "somewhat."

The present work does not support the above conclusions.

Figure 1 shows the physiological changes taking place in the leaves of one representative variety of winter wheat during a prolonged period of controlled low-temperature hardening. Temperatures

necessary to kill approximately 20 percent of the plants under a controlled 24-hour freeze are indicated at various stages of the hardening period. It is apparent from figure 1 that at least 7 weeks of continuous low-temperature ( $2^{\circ}\text{C}.$ ) hardening were necessary before the maximum hardiness was attained. Dry-matter and sucrose content increased throughout this period as did the resistance to freezing. Reducing sugars as glucose reached a maximum during the first 2 to 3 weeks of hardening and showed little quantitative change from then on. The actual increase in cold resistance appears to be correlated best with changes in dry-matter and sucrose content.

The changes taking place under artificial hardening seem closely comparable to changes which may be observed under natural field conditions. When the seedlings are placed in the hardening room growth is greatly retarded. The leaves gradually become "crisp" and assume a darker green color. Growth habit becomes progressively more decumbent as the peak of hardiness is approached until the plants finally assume a type of growth practically indistinguishable from that of field-hardened material.

Figure 2 illustrates the decumbent habit of artificially hardened plants. The two plants at *A* are typical of well-hardened seedlings. For contrast, the plant at *B* was selected from similar plants which had passed through their dormancy and started active "spring" growth. Their roots and all dead material were removed from the plants illustrated in figure 2 before they were photographed.

Artificially hardened wheat plants closely resemble field material in physiological make-up and actual cold resistance. This was established by comparing crown analyses on comparable wheat plants both field and artificially hardened and both at their approximate maximum cold resistance. The field material contained 26.65 percent of dry matter and 9.11 percent of sucrose; the artificially hardened, 26.55 percent of dry matter and 11.57 percent of sucrose. In both cases the temperature necessary to kill 20 percent of the plants was of the same order, i. e., within a degree of  $-20^{\circ}\text{C}.$  From the foregoing it appears evident that artificial hardening is to a high degree comparable to the processes involved in natural field hardening.

#### LIGHT AND HARDENING

Several authors have established that light is necessary for the normal hardening of winter wheat plants. In addition, Dexter (5) has demonstrated that under conditions of constant low-temperature hardening of wheat greater cold resistance develops under a long than under a short day. Controlled hardening technic is well adapted to studies of this nature.

In the early fall three series of flats, each flat containing the same six varieties of winter wheat, were placed in the hardening room and held at three different levels of light intensity for 43 days. The different levels of light intensity were obtained by shading, and consisted of unshaded or "full light," slightly shaded or "intermediate light," and heavily shaded or "low light." At the end of the 43-day period of hardening several flats from each series were frozen for 24 hours at  $-15^{\circ}\text{C}.$  and the survival counted at the end of a 2-week recovery period. The average survival of the six varieties was 84 percent for the "full light," 64 percent for the "intermediate light," and 11 percent for the "low light" series.

Samples of the leaves of the Minturki and Blackhull varieties from unfrozen flats under "full" and "intermediate" light were analyzed



FIGURE 2.—Decumbent habit of artificially hardened winter wheat plants (A) compared to the erect habit of a plant in active growth (B).

for dry matter and sugars. The leaves of the plants under "low light" were so yellow and necrotic as to make their use for analysis inadvisable. Table 2 shows the results obtained. It is obvious that the

decreased ability to develop cold resistance under lowered light intensity is accompanied by a decreased elaboration of dry matter and sugars.

TABLE 2.—*The effect of two levels of light intensity during low-temperature hardening of wheat on the accumulation of dry matter and sugars*

Variety	Dry-matter and sugar content under—					
	Full light			Intermediate light		
	Dry matter	Sucrose	Glucose	Dry matter	Sucrose	Glucose
	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>
Minturki	20.28	3.15	0.46	17.40	1.17	0.26
Blackbull	18.95	2.13	.39	16.23	0.54	.14

Since radiation is important in the development of cold resistance, a diurnal variation in cold resistance would be anticipated; i. e., cold resistance should reach its peak in the afternoon, drop somewhat during the following night, and then climb again during the succeeding day. Martin (18) and Salmon (26) reported that they found a decided tendency for hardening cereal plants to be injured more by day freezing than by night freezing. The diurnal variation in carbohydrate reserve is illustrated in table 3. The data reported are the averages of crown analyses for three varieties of hardening winter wheat plants grown in soil in 1939 and for two series grown and hardened as sand cultures in 1940. These data show that an increase in dry-matter and sugar content during the day is followed by a decrease during the night. It is significant that the decrease is only to a point considerably above that of the morning before. The hardening process may then be expressed as a zigzag progression, the cold resistance on each successive morning, though not equal to the highest point reached the day before, being an effective increase over that of the morning before. This of course assumes optimum hardening conditions.

TABLE 3.—*Diurnal variation in the dry-matter and sucrose content of hardening winter wheat seedlings*

Culture medium and sampling time	Dry matter	Sucrose
	<i>Percent</i>	<i>Percent</i>
Soil culture:		
Mar. 23, 1939—9:00 a. m.	25.17	8.55
Mar. 23, 1939—5:00 p. m.	26.73	10.55
Mar. 24, 1939—9:00 a. m.	26.57	9.94
Sand culture:		
Mar. 15, 1940—9:00 a. m.	23.10	8.94
Mar. 15, 1940—5:00 p. m.	24.70	10.92
Mar. 16, 1940—9:00 a. m.	24.15	10.45
Mar. 16, 1940—5:00 p. m.	25.65	11.66
Mar. 17, 1940—9:00 a. m.	24.74	11.01

As regards the influence of varied length of day during continuous low-temperature hardening there was no pronounced effect. So far as could be determined hardening progressed with about the same effectiveness either under the increasing day lengths of late winter



and early spring or under the decreasing day lengths of fall and early winter. The same morphological and carbohydrate changes took place during either period, resulting in the development of approximately the same degree of cold resistance.

#### THE DEHARDENING PROCESS

It has been indicated above that by dehardening is meant the process whereby hardened winter-wheat seedlings are caused to lose a substantial part of their cold resistance by transferring them from low

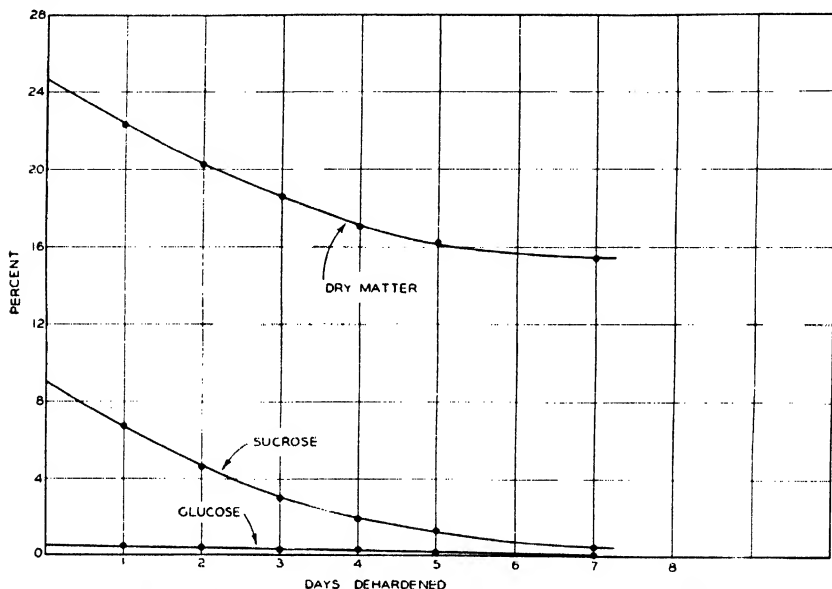


FIGURE 3.—Decrease of dry matter and sugars in the leaves of Minturki wheat during 7 days of high-temperature dehardening.

temperatures to the higher-temperature conditions optimum for active growth. Several investigators have demonstrated this loss of cold resistance under the influence of temperatures high enough to stimulate activity. Laude (14, 15) in particular has made an extensive study of the transition of winter cereals from dormancy to active growth. He concluded that transition as effected under favorable greenhouse conditions was essentially comparable to winter-spring transition in the field. Coincident with a progressive loss of cold resistance, he found an increase in water content and in the amount of expressed leaf juice, and a decrease in the total solid content of the sap. He likewise postulated resistance to loss of hardiness as a varietal characteristic.

Figure 3 shows typical curves illustrating the changes in dry-matter and sugar content when hardened winter wheat plants are placed under temperature and light conditions conducive to active growth. In this instance the analysis was on the "whole plant," only the roots and dead material being discarded. The plants had previously been held under controlled low-temperature hardening for 34 days and had

reached the level of physiologic hardiness indicated in figure 3 by zero days dehardening.

When the curves of figure 3 are compared to those of figure 1 it is obvious that there is a striking similarity between the two processes. Hardening is accompanied by an increase, dehardening by a comparable decrease, in dry-matter and sugar content. In other words, dehardening under these conditions may be described as a rapid reversal of the low-temperature hardening process.

#### ROLE OF LEAVES, CROWNS, AND ROOTS IN COLD RESISTANCE

Before any attempt is made to correlate composition with cold resistance, localization of the plant section responsible for survival is essential. Most investigators who have attempted to correlate chemical composition of winter cereals with cold resistance have used the leaves as analytical material. In fact, Newton and Anderson (23) stated that "the leaves are the organs which determine winter survival in wheat." The importance of the leaves in winter hardiness is emphasized by the defoliation experiments of Dexter (6) and Suneson and Peltier (33), who demonstrated that either artificial or natural defoliation interferes with the normal hardening process in winter wheat. It is only natural that leaf survival and hardening should be interrelated since the development of cold resistance apparently is associated with photosynthetic processes.

When, however, it is desired to compare varieties of winter wheat as regards cold resistance at any specific time during their hardening period, attention must be directed to this particular factor rather than to the general question of ability to harden. Martin (19) and Van Doren (38) found that the leaves of hardened winter wheat plants were less cold resistant than the crowns. Van Doren (38) likewise found a direct correlation between the bound water content of the crowns and cold resistance, this relation being just reversed for the leaves. Janssen (11) and Dexter (7) considered the crowns to be the seat of cold resistance and confined their analyses to this section of the winter wheat plant. Moreover, the work of Janssen (12) would suggest that the crown is the only organ involved in survival of hardened winter wheat plants. He removed both leaf blades and roots from field-grown plants during February and March and compared the development from the remaining crowns with the development of comparable untouched plants. He found some differences in growth but no decrease in final yield as a result of this drastic treatment.

In this investigation, likewise, it has been observed that the leaves have little if any role in the ability of hardened winter wheat seedlings to survive low temperatures. For instance, many comparisons in frost resistance were made between hardened plants which had been stripped of all their leaf blades immediately before freezing and duplicate undefoliated plants. No distinguishable difference was found in survival after the conventional 2-week recovery period. In all cases, even with 100-percent survival, the action of the low temperatures resulted in complete defoliation of the check plants, thus placing both sets on a comparable basis.

A more specific illustration was provided by the results of an experiment designed for another purpose. In a differential nitrogen nutrition experiment wheat was grown in sand culture, one part

receiving only ammonia nitrogen, the other exclusively nitrate nitrogen. After 3 weeks of growth the two series were hardened for 11 days, frozen for 24 hours at  $-15^{\circ}\text{C}.$ , and then removed to the warm greenhouse for recovery. The day following the freeze the plants of the nitrate series appeared to be badly damaged with all the leaf growth completely destroyed. On the other hand, the leaves of the plants in the ammonia series were green, crisp, and showed little injury. This condition persisted for several days after which period the leaves of the plants supplied only ammonia nitrogen turned yellow and killed back badly. About this time new leaves were beginning to arise from the crowns of the plants in the nitrate series. When the plants were examined for survival at the end of 2 weeks 75 percent of the plants of the nitrate series showed active new growth as compared to 11 percent for those of the ammonia series. This difference in behavior could not be attributed to root survival, as in this instance the plants of both series showed little root injury. If anything the plants of the ammonia series showed slightly less root injury than those of the nitrate series. On more detailed examination it was found that lack of growth was directly associated with the severity of crown injury.

Analytical data on crowns and leaves of different varieties of hardened winter wheat show that cold resistance is more closely correlated with crown composition than with leaf composition. This becomes very striking in some instances. An example is shown in table 4, where two varieties of soft red winter wheat are compared as to relative cold resistance of hardened seedlings and composition of comparable material. The dry-matter and sugar content of Kawvale crowns is considerably higher than that of Fulcaster crowns, thus showing agreement with relative cold resistance. On the other hand, the position is just reversed for the leaves; the dry matter and sugar content of these organs being higher in the less cold-resistant plants.

TABLE 4.—*Comparison of crown and leaf composition of two varieties of winter wheat with their relative cold resistance*

Variety	Freezing survival	Crown composition			Leaf composition		
		Dry matter	Sucrose	Glucose	Dry matter	Sucrose	Glucose
	Percent	Percent	Percent	Percent	Percent	Percent	Percent
Kawvale.....	100	20.50	6.18	0.72	21.70	2.24	0.31
Fulcaster.....	67	19.10	4.96	.62	21.98	2.69	.34

The roots of winter cereals have received very little attention in considerations of cold resistance. Zacharowa (39) attempted to correlate root composition and cold resistance of rye plants. Lamb (13) made an extensive study of the varietal characteristics of wheat roots as related to winter injury, particularly by soil heaving. Suneson and Peltier (31) noted that the roots of field-hardened winter wheat plants were less cold resistant than the crowns. This was confirmed by the present authors for soil-grown, artificially hardened plants. In addition, the problem was further clarified by the results of differential nutrition experiments with sand cultures.

Many thousands of sand-culture-grown wheat plants have been hardened, frozen, and subsequently examined in detail for root, crown, and leaf injury. No correlation whatever has been found between root survival and plant survival. Many instances were noted in which complete killing of the roots was coincident with 100-percent survival of the plants associated with these roots. On the other hand, under certain nutritional treatments instances were noted where the tops of plants showed 100-percent killing in spite of excellent root survival.

The role of differential nutrition in cold resistance will not be considered in this paper. For the present purpose one series illustrating the relationship between root injury and plant survival is presented without any consideration of the nutritional significance. The data are presented in table 5. The different types of nutrition are represented only by letters. Plant survival is expressed as exact percent and root survival as six different levels from zero to 100 percent. The only conclusion permitted by the data is that, under these conditions, root injury is of no consequence in the cold resistance of the plants.

TABLE 5.—Comparison of plant survival with root injury for winter wheat grown and hardened under 11 different nutrient conditions indicated by letter

Nutrition	Plant survival	Root survival	Nutrition	Plant survival	Root survival
	Percent	Percent		Percent	Percent
A	81	0	G	59	20
B	81	80	H	55	100
C	77	40	I	38	20
D	71	60	J	38	60
E	69	60	K	16	80
F	59	60			

<sup>1</sup> "Root survival" is based on observed frost injury, 100-percent survival indicating no injury and 0 indicating complete killing. Root injury was estimated on the basis of the degree of discoloration and tissue break-down when the plants were examined for survival 2 weeks after freezing.

The above data suggest that the cold resistance of wheat roots is of slight consequence in the survival of the frozen wheat plant. Accordingly, little attention has been directed towards the determination of the carbohydrate reserves of these organs. For incidental information a few analyses were made on the roots of hardened wheat plants. The average dry-matter and sucrose content for the roots of the plants in five series under differential nutrition was 15.05 percent and 3.75 percent, respectively. Analyses of the crowns of the same plants showed the dry-matter and sucrose content of these organs to be 28.57 percent and 12.42 percent, respectively.

#### VARIETAL DIFFERENTIATION

The development and maintenance of cold resistance, while not the only factor governing the winter hardiness of wheat, is in many areas the chief one. Even when considering this one factor of low-temperature hardening several modifying factors must be taken into account. The relative winter hardiness of a particular variety will depend not only on the maximum cold resistance which it can attain but also on the speed with which it can reach this level and its resistance to dehardening influences. The importance of the leaves in the hardening process should not be underestimated. It is their

function to carry on the metabolic processes leading to the development of cold resistance in the crown tissue. It is, then, essential that the leaves develop sufficient cold resistance so that their survival may permit the fulfillment of this function.

Figures 4, 5, 6, and 7 show graphically certain composition changes taking place in the six previously described varieties of winter wheat when subjected to uniform hardening and dehardening influences. In all cases both the glucose and sucrose content are expressed as percents of the water present in the plant tissue. The data will be considered independently as experiments A (fig. 4), B (fig. 5), and C (figs. 6 and 7), and then in relation to each other.

The material of experiment A was grown in the usual manner in the warm greenhouse and then transferred to controlled low-temperature hardening conditions on October 27, 1937. Several flats of Tennessee winter barley and of Marquis spring wheat were included for comparison. As a consequence of unseasonably warm weather during the early growth stage, it proved impossible with this one series to maintain temperatures as low as the desired 67° to 70° F. This resulted in a rather rank growth and necessitated a 4-week growth period before the desired uniformly tillered seedlings were obtained. Consequently when subjected to hardening the seedlings were a week older, larger, and more erect than those of experiments B and C.

After 30 days of controlled hardening, several flats of the material in experiment A were frozen at -15° C. for 24 hours and survival determined after a recovery period of 2 weeks in the warm greenhouse. The percentage survival for the six winter wheat varieties was Minhardi 86 percent, Minturki 90, Cheyenne 78, Kawvale 74, Blackhull 61, and Fulcaster 59 percent. These data show fair agreement with the field response as given in table 1 and indicate that the varietal response to controlled hardening approximates average field survival. Winter barley and spring wheat, hardened and frozen in an identical manner with the above treatment, showed zero survival.

Analyses were made on the leaves at intervals during a 44-day hardening period. As may be seen from figure 4, the behavior of this material was quite erratic. However, certain general trends may be established. Dry-matter content increased regularly and rapidly during the first 10 to 20 days of hardening, after which time the rate of increase was somewhat less pronounced. Reducing sugar content had a similar trend. The curve for sucrose content shows very little increase during the first 9 days, a very rapid increase during the next few days, and a somewhat slower rate of increase during the remainder of the hardening period. As far as varietal trends are concerned the correlation with known cold resistance or with field-exhibited winter hardiness is poor except that the dry-matter and sugar curves for the three most cold-resistant varieties are considerably higher than for the three varieties of lesser cold resistance. Freezing data showed separation into three groups: Minhardi and Minturki, high; Cheyenne and Kawvale, intermediate; and Blackhull and Fulcaster low. The pronounced lack of ability of the winter barley and spring wheat to attain any high level of reserves agreed well with observed freezing reaction.

After 49 days of hardening a number of the flats of experiment A, similar to the flats used to obtain hardening data, were removed to the

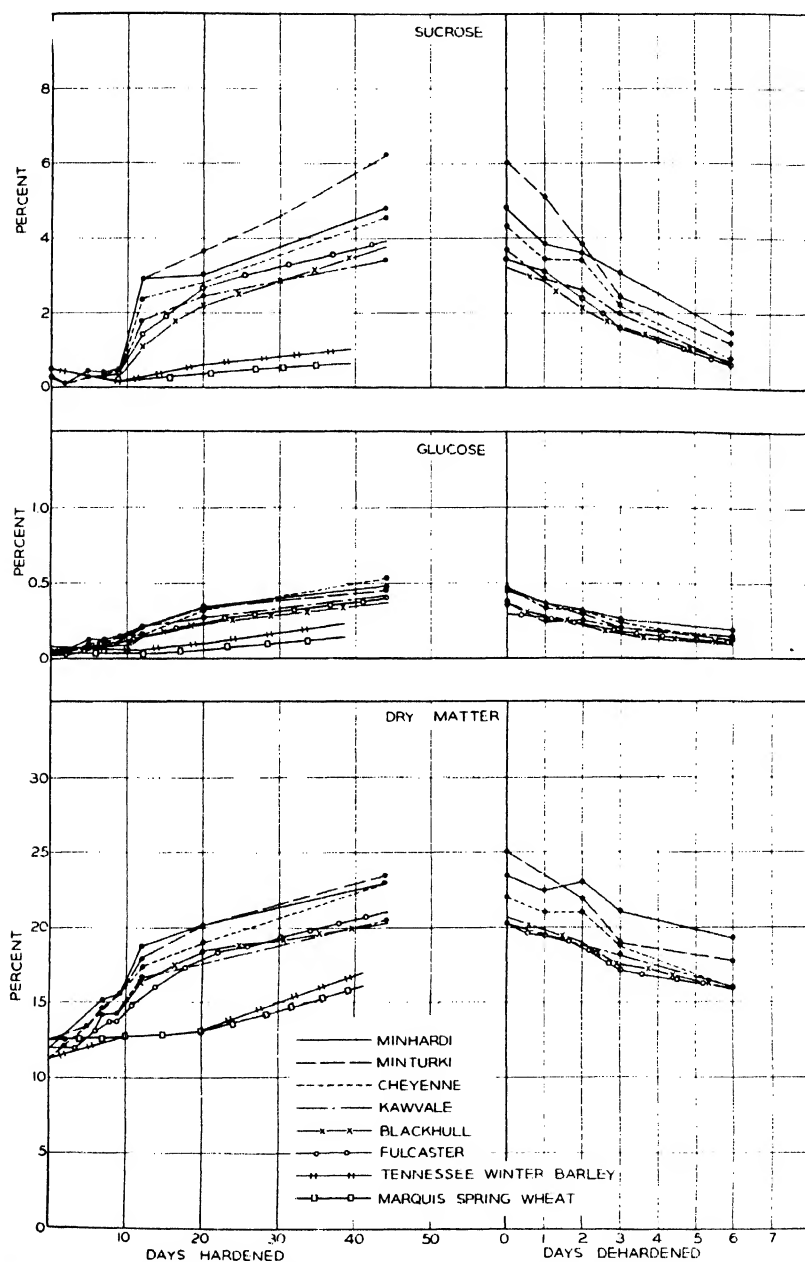


FIGURE 4.—Increase and decrease, during hardening and dehardening, of dry matter and sugars in the leaves of six varieties of winter wheat hardened during the period October 27 to December 10, 1937. Samples taken after 0, 2, 5, 7, 9, 12, 20, and 44 days of hardening and after 0, 1, 2, 3, and 6 days of dehardening. Experiment A. (Tennessee winter barley and Marquis spring wheat are shown for comparison.)

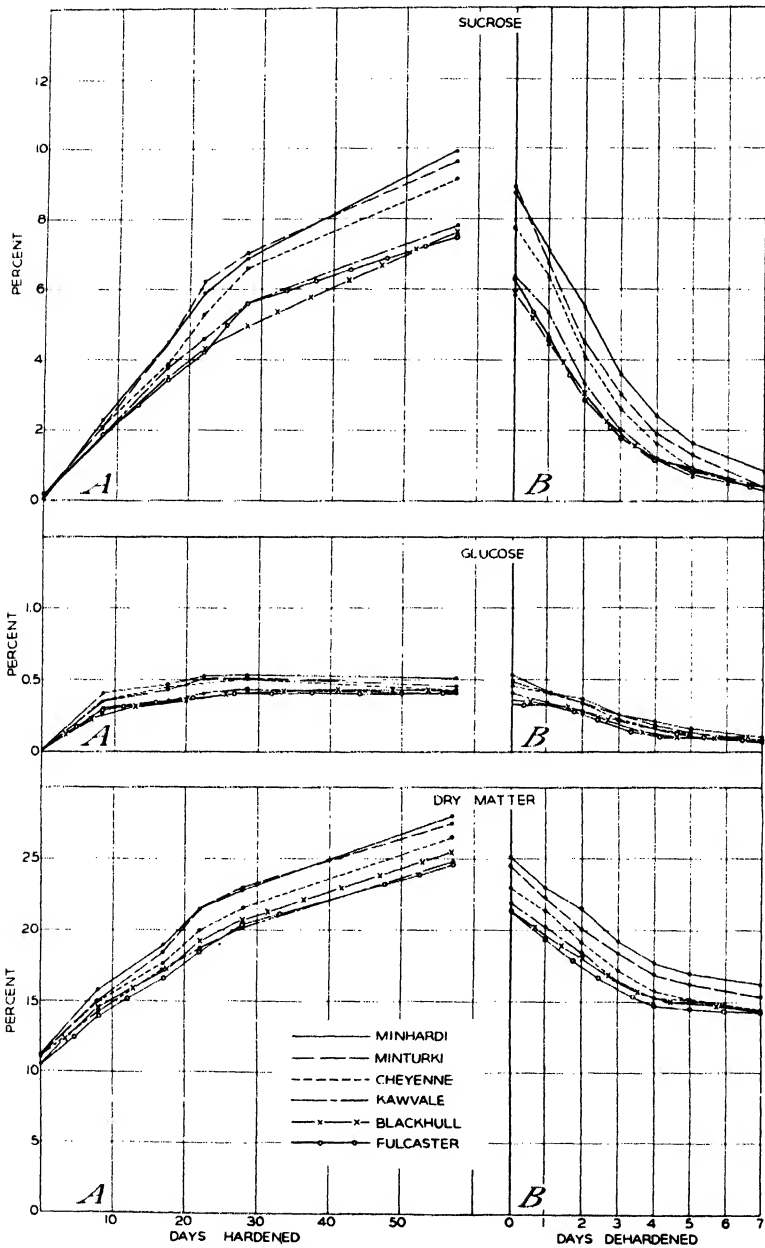


FIGURE 5.—Increase and decrease, during hardening and dehardening, of dry matter and sugars for the leaves (*A*) and whole plants (*B*) of six varieties of winter wheat hardened during the period December 13, 1937, to February 8, 1938. Samples taken after 0, 8, 17, 22, 28, and 57 days of hardening, and after 0, 1, 2, 3, 4, 5, and 7 days of dehardening. Experiment B.

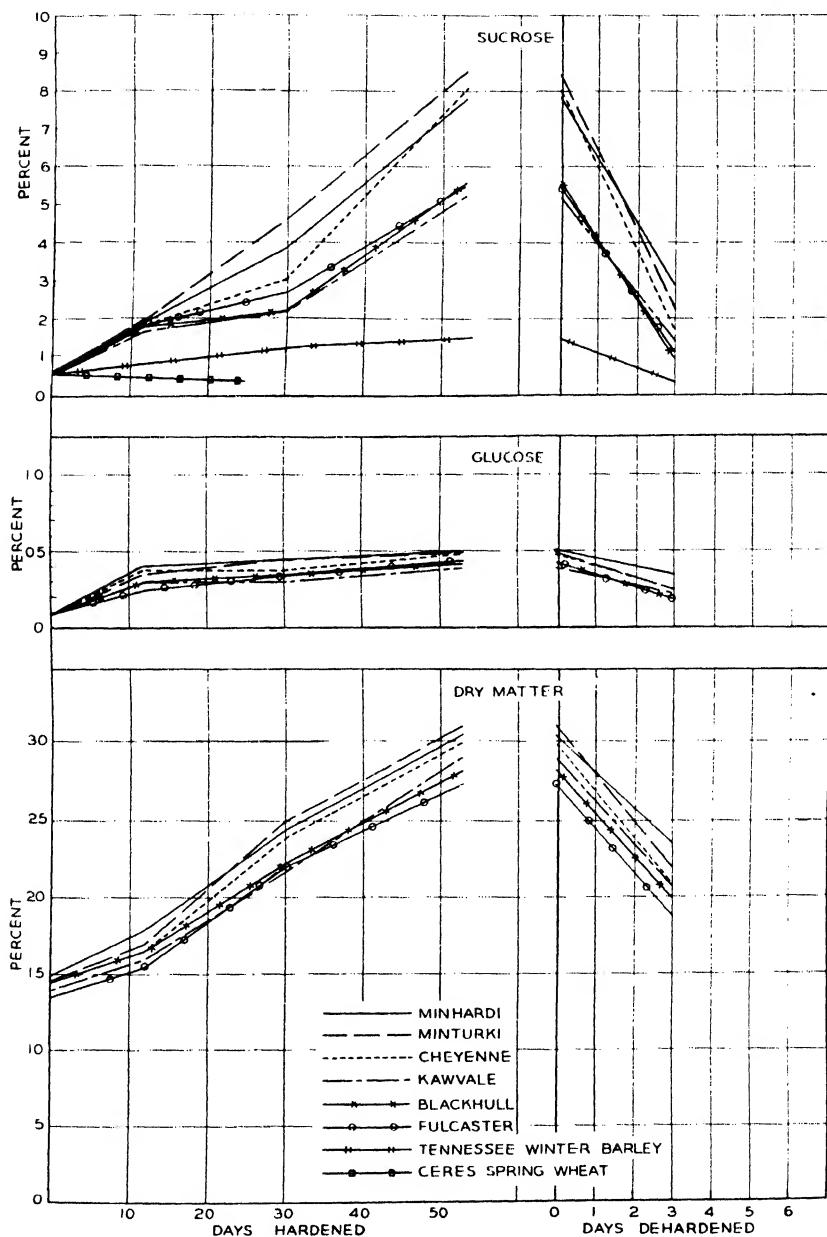


FIGURE 6.—Increase and decrease, during hardening and dehardening, of dry matter and sugars in the leaf blades of six varieties of winter wheat hardened during the period February 10 to April 4, 1938. Samples taken after 0, 12, 30, and 53 days of hardening, and after 0 and 3 days of dehardening. Experiment C. (Tennessee winter barley and Ceres spring wheat are shown for Comparison).



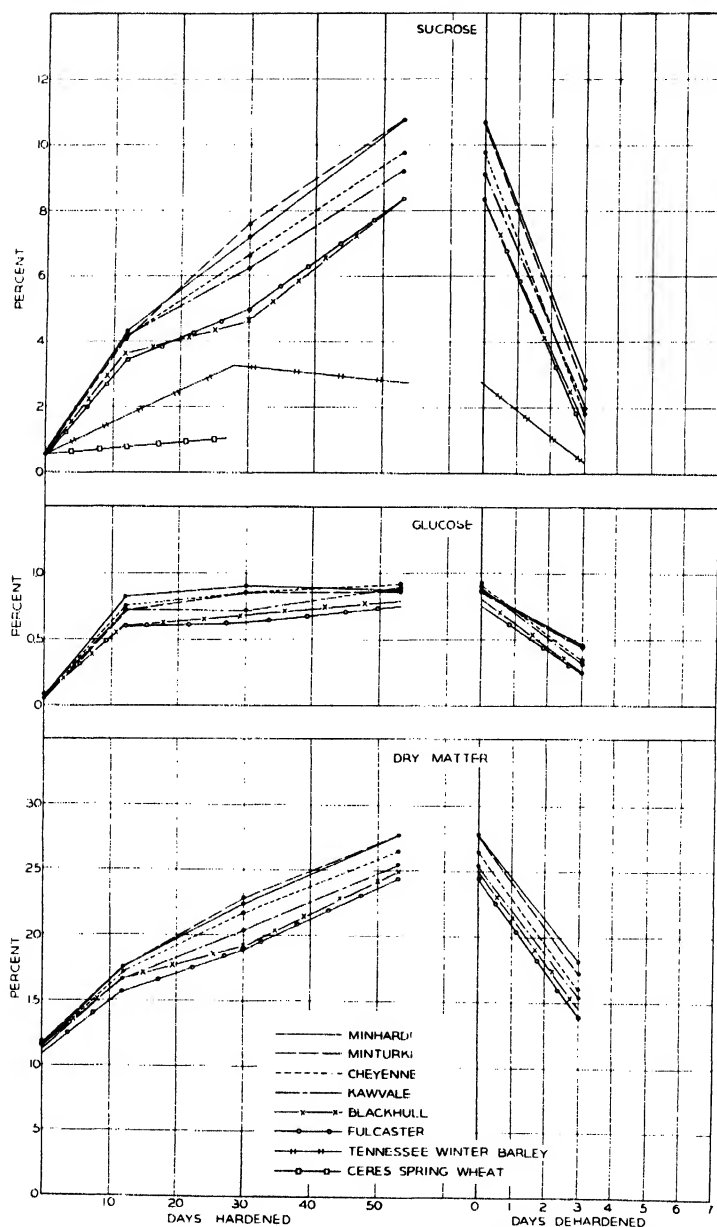


FIGURE 7.—Increase and decrease during hardening and dehardening of dry matter and sugars in the crowns of six varieties of winter wheat hardened during the period February 10 to April 4, 1938. Samples taken after 0, 12, 30, and 53 days of hardening, and after 0 and 3 days of dehardening. Experiment C. (Tennessee winter barley and Ceres spring wheat are shown for comparison.)

69° F. greenhouse and leaf analyses made at intervals during a 6-day dehardening period. As with the hardening plants, the nature of the growth resulted in unavoidable sampling errors and erratic results. However, definite and significant trends may be seen from figure 4. There is the usual progressive loss of reserves. However, the leaves of Minhardi and Kawvale resisted this loss to a pronounced degree. The net result is that the effect of dehardening was to orient the varieties in approximately the same manner as is observed in field behavior. Even after 6 days of dehardening the two most winter-hardy varieties retained their reserves to a relatively high degree.

Experiment B was carried out with the same six varieties of winter wheat as previously described. The seed was planted November 22, 1937, and grown for three weeks at 67° to 70° F. At the end of this period the seedlings were in good condition for hardening and analysis, i. e., actively tillered and exhibiting a uniform slightly decumbent growth habit. The flats were then removed to the hardening room and leaf samples taken for analysis at intervals during a 57-day hardening period.

As a check on the relative cold resistance of the wheat varieties several flats of material in experiment B were frozen after 37 days of hardening. A freezing treatment of -17.5° C. for 24 hours followed by a 2-week recovery period resulted in the following percentage survivals: Minhardi 62, Minturki 62, Cheyenne 50, Kawvale 47, Blackhull 13, and Fulcaster 12 percent. These data confirm those found for the material of experiment A inasmuch as the relative cold resistance of the varieties during controlled hardening is closely comparable to known average winter-hardiness response.

Data for dry matter, glucose, and sucrose in the plants of experiment B are presented in figure 5. The leaves of these plants showed a progressive accumulation of dry matter and sucrose throughout the whole hardening period. On the other hand, reducing sugars accumulated rapidly during the first few days, increased slowly up to about 4 weeks of hardening and then remained fairly constant for the remainder of the 8 weeks. The hardening curves show that some degree of varietal orientation is apparent almost from the initiation of hardening, this orientation becoming more pronounced with time. There is again the tendency for the varieties to segregate into two groups instead of the three groups indicated by freezing experiments. This is the result of the leaves of the Kawvale variety having about the same content of sucrose and dry matter as those of Fulcaster. As pointed out in table 4, the leaf composition of these two varieties tends to be similar, regardless of the higher degree of cold resistance exhibited by the Kawvale plants.

After 34 days of hardening a number of flats were removed to the 69° F. greenhouse and sampled at intervals over a 7-day dehardening period. The whole plant, less only the roots, was used for analysis. The data are recorded in the dehardening curves of figure 5. These curves show the same smoothness as those for hardening. Two or three days of dehardening resulted in the analyses showing a close correlation with winter-hardiness response. In fact at the end of 2 days of dehardening the varieties lined up in the exact order of known field response. Minhardi, and to some extent Minturki, leaves showed resistance to dehardening.

Experiment C was carried out with the same six varieties of winter wheat as used for the previous experiments. Three weeks after the planting date of January 20, 1938, the flats were removed to the hardening room and hardened in the customary manner for 53 days. Samples were taken at 0, 12, 30, and 53 days of hardening. After 53 days of hardening several flats were dehardened for 3 days, sampling being done at the end of this period. All samples were divided into two fractions for analysis, namely, the leaf blades and the crowns, and analysis carried out on each fraction independently. Results for dry matter, glucose, and sucrose are recorded for the leaf blades in figure 6 and for the crowns in figure 7. The sucrose content of Tennessee winter barley and of Ceres spring wheat were included for comparison. Because of shortage of material no data on freezing survival was obtained for the hardening plants of experiment C.

The data of figure 6 again show a progressive increase in dry matter and sucrose over the whole hardening period, this increase being somewhat more marked in the later stages of hardening. The curves for glucose again show an initial accumulation followed by very little further increase. The tendency for the usual orientation into two groups is marked. Three days of dehardening resulted in a good agreement of analytical and winter-hardiness data, with Minhardi leaves showing the usual resistance to dehardening. As in experiment A, the leaves of both the winter barley and the spring wheat showed a pronounced lack of ability to accumulate sucrose.

The curves in figure 7 show a pronounced and progressive increase in dry matter and sucrose content in the crowns throughout the entire hardening period. Those for glucose show the same type of rapid initial increase followed by little further accumulation as observed for the leaves. Orientation into the three groups established by freezing experiments is apparent for sucrose content at 30 days of hardening and is maintained at 53 days of hardening. The curves for dry-matter content show fair correlation with cold resistance, especially at 53 days of hardening. Three days of dehardening changes the relative position only slightly but what change there is tends to give a higher degree of correlation between composition and winter-hardiness response, especially as regards dry-matter content. As with the leaves, the crowns of the barley and spring wheat seedlings showed little ability to accumulate sucrose. As might be expected, the winter barley was intermediate between the spring and winter wheat in this respect.

Comparing the leaf-blade and crown analyses for experiment C (figs. 6 and 7), it is obvious that the dry matter and content the sucrose content of the crowns are correlated to a greater degree with varietal field winter hardiness than are those of the leaf blades. In addition, the relative crown composition is in excellent agreement with the freezing response shown by these artificially hardened plants in experiments A and B. In this particular experiment the leaf blades exhibited a higher dry-matter content than the crowns throughout hardening and dehardening. On the other hand, the sugar content of the crowns was consistently higher than that of the corresponding leaf blades, sucrose alone reaching, in the hardest varieties, a concentration of over 10 percent calculated on the water basis, or over 25 percent of the dry matter present.

Direct comparison of the three hardening periods represented by experiments A, B, and C can be made only with respect to leaf composition. However, the results of experiment C suggest that the behavior of the leaves gives a fair indication of the magnitude of change taking place in the crowns. It is apparent from the data for the three experiments that the greatest accumulation of reserves occurred in the leaves of those plants hardened through the latter part of December, January, and the first part of February (experiment B). Very close behind were those plants hardened through February and March (experiment C). This difference seems to be the result of the more rapid hardening of the B plants during the first 30 days. In both cases the reducing-sugar content shows a similar response to low temperatures and cannot be considered of great significance in the general hardening picture with the possible exception of the early stage.

The plants showing the least ability to accumulate reserves were those hardened during November and the first part of December (experiment A). The lack of hardening ability, as well as the erratic results shown by this series, may be in part attributable to the nature of growth. The fact that the plants of experiment A were older, larger, and not possessed of the same desirable winter habit as those of the other two series makes the results of somewhat questionable significance. However, they do indicate the type of growth which is undesirable for controlled hardening and suggest that the hardening process is modified by the plant's growth habit. Under the constant low-temperature hardening conditions the nature of the response of the winter wheat plants to hardening under decreasing day lengths (experiment A) is little different from that under the influence of increasing day lengths (experiments B and C).

Crown analyses were made on the material of experiment B at 57 days of hardening only. At this stage of hardening the six varieties showed a range of from 27.65 to 24.43 percent dry matter, from 12.29 to 9.77 percent sucrose, and from 1.00 to 0.83 percent reducing sugars as glucose. Comparing these data with those of the experiment C crowns as recorded in figure 7, it is apparent that the plants hardened during late winter showed a somewhat greater accumulation of reserves than did those hardened during early spring. It is questionable whether or not this difference is sufficient to indicate any greater efficiency of hardening during the somewhat shorter days prevailing when the plants of experiment B were grown and hardened.

The data of experiments A, B, and C indicate that Minhardi plants show resistance to loss of reserves when the plants are subjected to dehardening conditions, this being particularly true for the leaves. That this is likewise true for cold resistance was demonstrated by freezing a number of flats of dehardening plants. The material used was part of experiment C and was that remaining when this experiment was terminated. At this time, early April, high outside temperatures made impractical the maintenance of sustained low temperatures in the hardening room. During the succeeding 3 weeks refrigeration was so manipulated that day temperatures were maintained at 65° F., and night temperatures gradually increased until at the end of this period they, too, approximated 65° F. Several flats of material were frozen after 10, 16, and 23 days of this dehardening treatment. A constant temperature and period of freezing was used, namely, -17° C. for 24 hours. The results are given in table 6.

TABLE 6.—*Cold resistance measured by survival of 6 winter wheat varieties, frozen at  $-17^{\circ}$  C. for 24 hours, after 10, 16, and 23 days of slow dehardening*

Days dehardened	Survival of the 6 varieties					
	Minhardi	Minturki	Cheyenne	Kawvale	Blackhull	Fulcaster
	Percent	Percent	Percent	Percent	Percent	Percent
10	97	95	95	92	25	17
16	92	72	57	65	3	13
23	60	9	3	8	0	0

The data of table 6 show that the Minhardi variety has pronounced resistance to dehardening influences. Even after 23 days of dehardening, when Blackhull and Fulcaster were completely killed and Minturki, Cheyenne, and Kawvale showed less than 10-percent survival, 60 percent of the Minhardi plants were in active growth after a 2-week recovery period. The results clearly indicate that the resistance which the Minhardi variety shows to loss of reserves during dehardening coincides with an ability to retain cold resistance.

#### DISCUSSION AND CONCLUSIONS

Climatic conditions vary widely in different regions of the winter wheat growing area of the United States. Considering winter hardiness only from the cold-resistance aspect, it is obvious that the requirements for a variety to be winter hardy in a southerly latitude are not necessarily the same as those permitting winter survival in a region farther north. For example, let us postulate a theoretical "winter" climate where early fall frosts are to be anticipated, where extremely low winter temperatures are exceptional, and where frequent warm periods alternate with cold throughout the winter. A variety with favorable winter hardiness for this specific climate should rapidly attain a degree of cold resistance in the fall, and while a high level of cold resistance is not necessary at any period, resistance to the dehardening influence of high temperatures is essential. Modification of the climatic conditions postulated above necessitates modification of the winter-hardiness characteristics which would qualify a variety as ideally adapted. The difficulty of observing such varietal characteristics in the field suggested the present studies in which the reaction to controlled hardening and dehardening conditions may be determined.

When winter wheat plants were grown with uniform soil conditions and hardened under exactly comparable conditions of low temperature and light, increases in dry matter and sucrose content were coincident with increases in cold resistance. Winter wheat varieties which showed a high level of cold resistance in the hardened condition and which exhibit a high degree of winter hardiness in the field also proved capable of building up higher dry-matter and sucrose content under controlled hardening than did those varieties of lesser cold resistance and winter hardiness. Whether the increase in sugar content during hardening confers cold resistance or is simply a result of conditions leading to the development of cold resistance is a debatable question. Certainly other physiologic changes likewise coincide with hardening. Too, it should not be assumed that under all growth conditions sugar and dry-matter content will be a reliable index of cold resistance. It has been demonstrated <sup>3</sup> that variation in nutrition may destroy this relationship.

<sup>3</sup> Unpublished data.

Temperature and light intensity are unquestionably major factors influencing the development of cold resistance in winter wheat. Hardening is likewise dependent upon such supplemental factors as the preceding stage and nature of growth. Since the response to temperature is a varietal characteristic, there is some justification for believing that the response to light intensity and supplemental factors may likewise be varietal in nature. Further, the studies show that the temperature response itself need not be the same for all parts of the plant. For instance, the leaves of one variety may show more ability to accumulate reserves than those of another, while the response of the crowns may be just reversed. While the cold resistance of the crown governs survival of the plant, it is obvious that leaf survival is of importance especially during the period when metabolic processes are gradually building the high levels of reserves attained by midwinter.

The foregoing observations help to explain the reason for the "reversals" often encountered in winter-hardiness nurseries. When two varieties of winter wheat are compared relative to their winter hardiness, as determined over a number of years, one variety may be found decidedly superior to the other. However, in certain years this position is reversed, the variety of high average winter hardiness being abnormally low. In view of the fact that climatic factors vary widely from year to year and that varietal winter hardiness must be dependent on such factors as speed of hardening, level of maximum attainable cold resistance, resistance to dehardening, response to variable light conditions, and others, it would be surprising if such reversals did not occur.

Controlled hardening studies, not being dependent on variable climatic conditions, should provide a means of predicting the average field behavior of a variety. The present study indicates that this cannot be done merely on the basis of the relative content of carbohydrate reserves at any one time during hardening. For example, the relatively great field winter hardiness of Minhardi wheat may be the result not only of its ability to acquire a high level of cold resistance but likewise the result of rapidity of hardening and ability to retain cold resistance under dehardening influences. On the other hand, the low degree of winter hardiness which such varieties as Blackhull and Fulcaster exhibit in the field would be expected by reason of their lack of ability to develop a high level of reserves.

The data presented would seem to justify the use of sucrose or dry-matter content, or both, as a measure of the changes in cold resistance taking place under controlled uniform hardening or dehardening conditions. The use of controlled hardening in conjunction with either adequate freezing or chemical analysis should provide a valuable adjunct to nursery and controlled freezing techniques in the determination of varietal winter hardiness.

#### SUMMARY

Controlled low-temperature hardening technique in conjunction with high-temperature dehardening was applied to the study of winter hardiness in wheat. Degree of cold resistance at any stage of hardening was measured either by controlled freezing or by analysis for dry-matter and sugar content. The ferrieyanide method for sugar deter-

mination was successfully applied in a form permitting great rapidity of analysis.

The progressive increase of dry-matter and sucrose content over an 8-week controlled low-temperature hardening period was demonstrated. A parallelism was established between these increases and increases in cold resistance. Controlled hardening was shown to be comparable to natural field hardening, the morphological and physiological changes in the wheat plant being very similar for both. Light intensity was found to be a very important factor in hardening. Diminution of light intensity resulted in decreased efficiency of hardening as regards both cold resistance and storage of reserves. Under constant low-temperature conditions photoperiod was apparently not an important factor as plants hardened adequately under either increasing or decreasing day-lengths. Growth stage preceding hardening influenced the efficiency of hardening.

High-temperature dehardening, i. e., the loss of cold resistance during the period of transition from dormancy to active growth, proved essentially a reversal of the hardening process. Dry-matter and sugar content showed a progressive decrease with time of dehardening.

The cold resistance of neither the roots nor the leaf blades is a reliable index of the cold resistance of the plant. Survival seems to be entirely dependent on the ability of the crown to resist frost injury. However, the importance of leaf survival as contributing to the physiologic changes associated with the acquisition of cold resistance is evident.

Controlled low-temperature hardening and high-temperature dehardening studies were made on six varieties of winter wheat with the occasional inclusion of a variety of winter barley and one of spring wheat. Response to these conditions proved varietal in nature. There was good agreement between relative field-exhibited winter hardiness and the development of cold resistance and accumulation of reserves under controlled hardening. This was particularly true for the plant crowns. Leaf composition showed fair agreement with known cold resistance, this agreement being enhanced by a 2- or 3-day dehardening period. The studies suggest that observations of the trend of carbohydrate metabolism under the influence of controlled hardening and dehardening would prove of distinct value in determining the varietal winter hardiness of wheat.

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# STRAINS OF CUCUMBER MOSAIC VIRUS PATHOGENIC ON BEAN AND PEA <sup>1</sup>

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## INTRODUCTION

During a study of spotted wilt of garden pea (*Pisum sativum* L.) in the spring of 1935 (36),<sup>3</sup> an unusual development of symptoms atypical of that disease occurred in an out-of-door planting of peas at Madison, Wis. Mild mottling appeared on the younger leaves as well as a faint necrosis on stems, leaves, and pods. When juice extracted from certain of these plants was transferred to tobacco (*Nicotiana tabacum* L. var. Havana 38), local and systemic symptoms of spotted wilt developed on a small percentage of the plants. On the remainder of the plants either local necrotic lesions or chlorotic areas occurred on the inoculated leaves, but the systemic mottle which followed was definitely not characteristic of the disease in question. Moreover, a contaminating virus was demonstrated by means of differential hosts. The mottle virus was isolated by inoculation to cucumber (*Cucumis sativus* L. var. White Spine) and the spotted wilt virus by inoculation to nasturtium (*Tropaeolum majus* L.).

Adjacent to the planting of peas was a number of rows of bean (*Phaseolus vulgaris* L.) which included the varieties Wisconsin Refugee and Idaho Refugee, known to be resistant to common bean mosaic (bean virus 1). As these plants came into blossom, about 5 percent of them showed symptoms similar to yellow bean mosaic (bean virus 2) (21). As the season progressed, however, the symptoms differed in many respects from those of the latter disease. In addition to marked stunting and various degrees of mottle, necrosis occurred on leaves, stems, and petioles. Some resemblance to bacterial blight (*Bacterium phaseoli* EFS) was occasionally suggested, but attempts to isolate this bacterial organism yielded negative results. Inoculation with expressed juice to pea, bean, cucumber, and tobacco resulted in mosaic on the last two species, while on pea the symptoms were mild necrosis and faint mottle and on bean a diffuse mottle and severe stunting.

These preliminary cross inoculations suggested that the diseases on pea and bean were possibly due to the same virus and that the latter was capable of bringing about symptoms on cucumber and tobacco not unlike those associated with cucumber virus 1. In the autumn of the same year (1935), M. W. Stubbs brought to the writers from a Madison greenhouse a young pea plant which showed mild stem necrosis. Inoculation to cucumber, tobacco, and pea resulted in infection on all three, while transfer to bean was unsuccessful. Since none

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<sup>3</sup> Italic numbers in parentheses refer to Literature Cited, p. 58.

of the mosaic or streak viruses heretofore isolated from pea or kidney bean, except the tobacco-ring-spot virus, had been shown to infect systemically both tobacco and cucumber, it was deemed essential to give the matter further attention. The present paper is a report on studies of the diseases and associated viruses which have been carried on at the Wisconsin Agricultural Experiment Station since 1935. A preliminary report (37) has already been published.

#### METHODS AND MATERIALS

The greenhouses in which studies were conducted were kept free of insects by frequent fumigation. In most cases the experimental plants were grown at temperatures most favorable for their normal development, and after inoculation they were held at 23° to 26° C. Insect transmission studies were conducted in separate compartments. Field symptoms on peas, beans, cucumbers, and squash (*Cucurbita pepo* L. var. Giant Summer Crookneck) were studied on experimental plots near Racine, Wis., during the summer of 1939.

Preliminary investigations were carried out with a number of isolates<sup>4</sup> from pea and bean. Since most of these isolates were similar, one was selected for further study as representative of those infectious to both pea and bean. This virus was obtained from a necrotic pea pod of the Wisconsin Perfection variety. The diseased plant was grown in an outdoor plot at Madison, Wis., and showed a necrotic flecking of the leaves and streaking of the stem. This isolate will be referred to as strain 14. The other strain used extensively in this investigation was that secured from a necrotic pea plant, mentioned above, collected in a Madison greenhouse by M. W. Stubbs. This is referred to as strain 17, and at the outset it was distinguished from strain 14 by the fact that it was not infectious to bean. Certain sub-strains of 14 were isolated for study; the procedure used is described later in the text.

For comparative purposes a stock of cucumber virus 1 was obtained from James Johnson, Wisconsin Experiment Station. Celery virus 1 was supplied by F. L. Wellman, United States Department of Agriculture; it was isolated originally by him from *Commelina nudiflora* L. in Florida. All stocks of the viruses were maintained in young hybrid tobacco plants (*Nicotiana tabacum* × *N. glutinosa* L.), those for each virus being segregated on shelves widely separated from other plants.

All greenhouse plants were inoculated in early stages of growth. Peas were more readily infected when the first leaves were inoculated before they had unfolded, while beans were usually inoculated by rubbing the unifoliate leaves about the time when the first trifoliate leaf was unfolding. Carborundum powder was used as an abrasive in transfer of the viruses, and the residue was washed from the rubbed leaves with a fine spray. This method was used in all studies of symptoms, host range, and properties.

#### EXPERIMENTAL RESULTS

##### REACTION OF BEAN

In greenhouse studies strain 14, strain 17, cucumber virus 1, and celery virus 1 were used to inoculate the following varieties or strains

<sup>4</sup> The term "isolate" is used in this paper to designate a virus from a given source without implying that it is necessarily a distinct strain.

of bean: Asgrow Stringless, Black Valentine, Stringless Black Valentine, Asgrow Valentine, Tendergreen, Early Stringless Green Pod, Giant Stringless Green Pod, Full Measure, Bountiful, French Horticulture, Ruby Dwarf Horticulture, Tennessee Green Pod, Wisconsin Refugee, Idaho Refugee, Corbett Refugee, Kentucky Wonder Pole (white-seeded), Navy White, Kentucky Wonder Pole (regular), Sure Crop Black Wax, Hodson Wax, Brittle Wax, Improved Kidney Wax, Pencil Pod Black Wax, Wardwell Kidney Wax, and Keeney Golden Wax. At least 2 series, each containing 5 to 25 plants, were inoculated from each variety. All varieties inoculated with strain 14 developed symptoms. This virus was successfully recovered by transfer to young tobacco plants of juice extracted from each of 16 varieties selected at random. Many attempts to recover the other 3 viruses were unsuccessful.

It is evident that strain 14 is distinctive in that it is the only one of the four viruses used which affects bean. Wellman (33) attempted to infect bean with celery virus 1, but likewise secured negative results. Harter (7) described a virus (believed to be related to cucumber virus 1) that was pathogenic on several varieties of lima bean (*Phaseolus lunatus* L.), but not on kidney bean or pea. The preliminary report by the writers (37) is apparently the first record of the successful inoculation of a virus in the cucumber mosaic group to kidney bean and pea. More recently Zaunmeyer (39) has reported two strains of cucumber virus affecting pea, sieva lima bean, and broadbean (*Vicia faba* L.), but not kidney bean.

Although no variety was found immune to strain 14, some differences in expression of symptoms and in the amount of stunting, mottling, and necrosis occurred. In general, these symptoms were not sufficiently distinct to warrant placing the varieties in different classes as suggested by Pierce (21) for bean virus 1. The range of symptoms may be said to include about all those described for both bean virus 1 and bean virus 2. On the other hand, it is difficult to ascribe to strain 14 any one symptom or group of symptoms which might be sufficiently characteristic to set it apart from the other two viruses just mentioned. Four varieties representing the range of symptoms were selected for detailed study.

The unifoliate leaves of the Wisconsin Refugee develop a distinct drooping within 4 or 5 days after inoculation with strain 14. If these primary leaves are not fully developed at the time of inoculation, vein clearing and yellowing are also produced in them. The first trifoliate leaf next shows spinasty and vein clearing, with each leaflet twisted and curved slightly inward from the apex. As the leaf continues to develop, a diffuse yellow mottle appears, while the second trifoliate leaf develops a more distinct mottle of the same type (fig. 1, C).

The drooping of the unifoliate leaves serves to distinguish strain 14 from bean virus 2, which Pierce (21) described as causing a downward drooping of the first trifoliate. Since bean virus 1 does not affect the position of the leaves, epinastic response may be used to distinguish each of the three viruses in the early stages of development on plants inoculated in the simple leaf stage in the greenhouse.

Soon after the appearance of the first symptoms induced by strain 14, retardation in growth is evident (fig. 2). This difference becomes even greater with age and is frequently the chief means of distinguishing the diseased from the healthy plants. Retardation in develop-

ment of leaves may be seen in figure 1, *C, E*, which shows a healthy and a diseased leaf from Wisconsin Refugee plants of the same age. The petioles and the internodes are also shortened, and stimulation of axillary buds gives the plant a bushy appearance, especially under field conditions.

In the same field in which strain 14 was originally isolated certain Wisconsin and Idaho Refugee plants developed a light yellow mottle

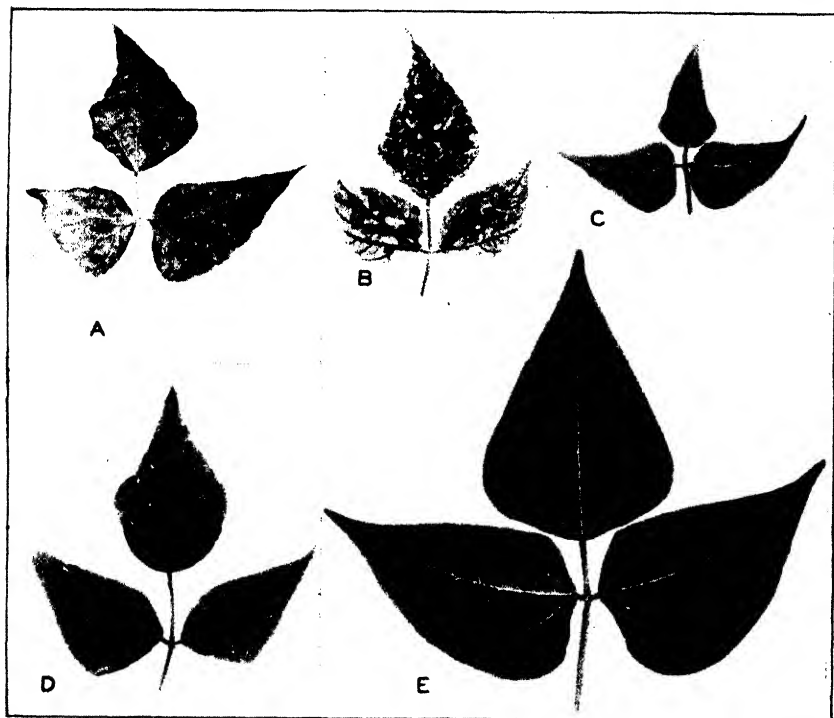


FIGURE 1.—Symptoms produced in 3 weeks on the second trifoliates of plants of four varieties of bean inoculated with strain 14: *A*, Mottling and wavy leaf margins in Hodson Wax; *B*, pronounced mottling, vein banding, and dark green islands in Sure Crop Black Wax; *C*, diffuse mottle, slight curling of apices, and extreme reduction in size in Wisconsin Refugee; *D*, chlorosis near margins and deeper than normal green in the region of the midvein in Full Measure; *E*, uninoculated leaf of Wisconsin Refugee. Note marked reduction in size of all inoculated leaves.

similar to that described for bean virus 2 (21). Isolations from these plants consistently produced symptoms on both tobacco and beans. Since under greenhouse conditions the inoculated bean plants later outgrew the leaf mottle symptoms, it was concluded that bean virus 2 was probably not present in these plants and that the field symptoms were due to strain 14. Other plants in this field showed necrosis of leaves, petioles, and stems, a symptom which was especially prominent at the pulvinus and growing tip. It is now believed that one or more yellow strains of 14 were present in this field, since these necrotic symptoms are similar to those later produced with such strains of the virus.

Further field trials were conducted during the summer of 1939 near Racine, Wis. Three 50-foot rows of Wisconsin Refugee were planted, and when the third trifoliate was unfolding the first 20 plants of each of two rows were inoculated with strain 14. One month later the infected plants showed marked stunting but had not developed a definite mottle. The only other signs of disease were the irregular glossy surface and vein clearing of younger leaves. An occasional uninoculated plant in each of the three rows also showed symptoms similar to those which had been inoculated. Transfer of inoculum from these plants to tobacco demonstrated that they were also infected with strain 14. By the end of the season 90 percent of the uninoculated plants had become infected. In no case was mottling or necrosis very evident; marked

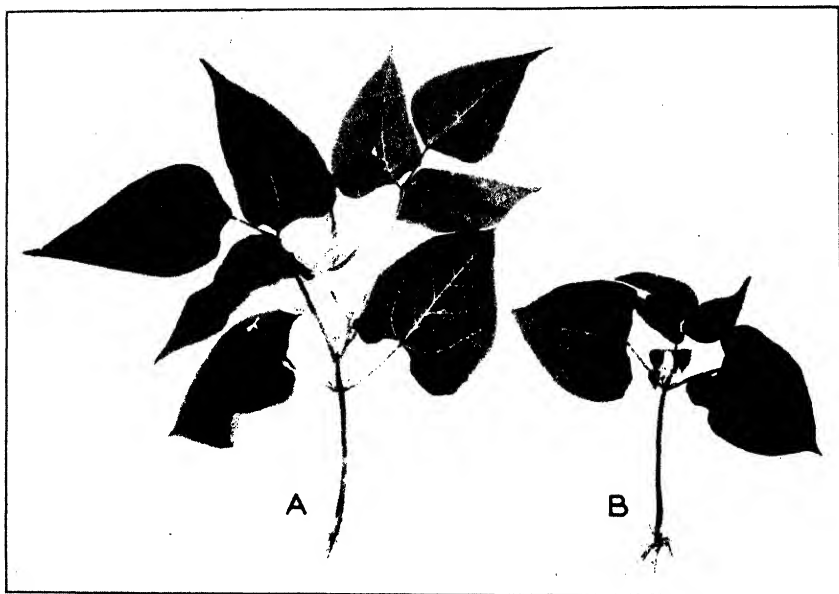


FIGURE 2.—A, Uninoculated Wisconsin Refugee bean plant; B, plant of same age as that in A, inoculated 3 weeks previously with strain 14. Note extreme retardation in growth.

stunting and the glossy, wavy appearance of the leaves were the only distinguishing symptoms. As compared with the few remaining healthy plants, the production of pods on these diseased plants was greatly reduced, and small misshapen pods were more common. The natural spread of the virus was probably brought about by aphids. The potato aphid, *Macrosiphum (Illinoia) solanifolii* (Ashm.), was abundant and an occasional peach aphid, *Myzus persicae* (Sulz.), was observed. Although the capacity of the potato aphid to transmit strain 14 has not been investigated, it is an established vector of cucumber virus 1, while *M. persicae* is shown later in this paper to be a vector of strain 14.

Considerable variation in symptoms has been shown to occur on Wisconsin Refugee in the greenhouse and in the field. Even greater variations occur between different varieties. On Sure Crop Black

Wax, symptoms are more pronounced than on any of the other varieties tested and they closely resemble those produced by bean virus 2. Initial symptoms are much like those produced on Wisconsin Refugee, with drooping of the unifoliates and vein clearing of the first trifoliates, the latter condition usually changing gradually to vein banding. Light yellow areas soon develop and gradually spread to all parts except along the larger veins, which remain dark green (fig. 1, *B*). Occasionally, the vein banding symptom does not persist and the mottle is more like that of bean virus 2, except that the yellowing is less extensive. Scattered small dark green islands commonly occur. The plants are stunted to about the same degree as those of Wisconsin Refugee.

On Full Measure the symptoms on the first and second trifoliolate often resemble those of bean virus 1, as described by Pierce (21) on Stringless Green Refugee. Vein clearing is followed by chlorosis, especially along the edges (fig. 1, *D*). On some leaves small dark green areas appear scattered promiscuously over the lighter green surface or concentrated along the larger veins. These symptoms may continue to appear on developing leaves, but usually become entirely masked. Stunting is less severe than in Wisconsin Refugee.

Symptoms on Hodson Wax are usually very mild under greenhouse conditions. The first and second trifoliates usually show a mild, diffuse mottle and occasionally darker green areas (fig. 1, *A*). On later-developing leaves it is often impossible to detect any mottling, and the leaf may show only an irregular, glossy surface. Stunting is less marked than with the varieties described above, and it is often necessary to transfer the virus to other hosts to ascertain infection.

In general, stunting is the most constant symptom of strain 14 on the bean varieties studied. The slower rate of growth causes a reduction in size of leaves and a shortening of internodes and petioles. Stunting varies with the variety, but it is a more constant feature than in the case of bean virus 1. Mottling of the leaves is extremely variable, depending on the environment as well as the variety. The slightly irregular, glossy surface is a rather constant feature but is too indistinct to be detected by the casual observer. Masking of leaf symptoms in the greenhouse and field is common. More extensive field observations are needed to determine the most constant field symptoms.

The reaction of the bean to various "yellow" and "dark green" substrains of 14 is discussed later in this paper.

#### REACTION OF PEA

Three varieties of pea were inoculated with the same four viruses used in the bean studies. The results (table 1) show that symptoms appeared in all cases except in the plants of the Wisconsin Perfection variety inoculated with cucumber virus 1 and celery virus 1. Furthermore, in the case of the Alderman variety local lesions only were produced after inoculation with celery virus 1. In no case was cucumber virus 1 or celery virus 1 recovered from inoculated pea plants even though symptoms had occurred. Strains 14 and 17, on the other hand, were recovered quite readily.

The symptoms which develop on young plants inoculated and grown in the greenhouse will be described first.

TABLE 1.—*Comparative symptoms on three varieties of pea inoculated with strain 14, strain 17, cucumber virus 1, and celery virus 1*

Variety	Strain 14		Strain 17		Cucumber virus 1		Celery virus 1	
	Plants inoculated	Plants infected	Plants inoculated	Plants infected	Plants inoculated	Plants infected	Plants inoculated	Plants infected
	<i>Number</i>	<i>Percent</i>	<i>Number</i>	<i>Percent</i>	<i>Number</i>	<i>Percent</i>	<i>Number</i>	<i>Percent</i>
Alderman.....	137	82	55	47	20	24	10	1 20
Alaska.....	74	62	28	50	12	28	14	21
Wisconsin Perfection.....	88	49	27	52	30	0	17	0

<sup>1</sup> Only local lesions were produced.<sup>2</sup> The only symptom was stem necrosis at the base of the inoculated leaf.

## STRAIN 14

When young pea plants of the Alderman variety are inoculated before the first leaves have unfolded, the initial symptoms appear in 4 or 5 days as a wilting or necrosis of some of the rubbed leaves and stipules. In 6 to 10 days there appear on the youngest leaves diffuse, yellow-green areas which may develop into a more definite mottle (fig. 3, *B*), or may disappear entirely. The developing leaves on infected plants tend to remain folded and slightly twisted. A pronounced stunting soon follows, with reduction in size of leaves and shortening of internodes as the most conspicuous symptoms (figs. 4 and 5). The lower leaves continue to wilt and die, and under greenhouse conditions 35 to 50 percent of the plants of this variety develop faint, but definite, watery, purple-brown, necrotic streaks on the stem. Necrosis may advance up one side of the stem or affect the entire plant, including leaves and growing tip. Leaf necrosis is usually most evident along the veins. If the growing tip is killed, new shoots may develop in the axes of the lower leaves, but they remain stunted and often show mottling. Infected greenhouse plants may persist for some time but eventually die prematurely.

On the Alaska variety symptoms are similar to those on Alderman except that stem necrosis is more extensive. Symptoms on Wisconsin Perfection frequently appear as diffuse necrotic areas on the leaflets and occasionally as a streaking on the petioles and stem. Stem necrosis is more pronounced and mottling more distinct than on the other two varieties. The killing of the growing tip is also common. Symptoms produced by substrains of the virus on this variety are described later in this paper.

In the field two 50-foot rows each of Alderman and Wisconsin Perfection were planted, and 4 weeks later 20 plants in one row of each variety were inoculated mechanically with strain 14. Mottling of some plants and stunting of others were evident in 2 weeks, and many later developed a mild necrosis of the stem and growing tip. At 5 weeks all inoculated and a few uninoculated plants had developed the disease. By the end of the season 95 percent of all the plants were infected, and isolations from representative plants gave only strain 14. Since the potato aphid could be found on almost every plant and the peach aphid on an occasional one, it is probably that one or both of these species spread the virus from the artificially infected plants.



Initial symptoms on naturally infected plants were similar to those described above except that death did not often occur as promptly. However, the main shoots usually died and secondary branches developed near the base of the plant. The rosettelike appearance of

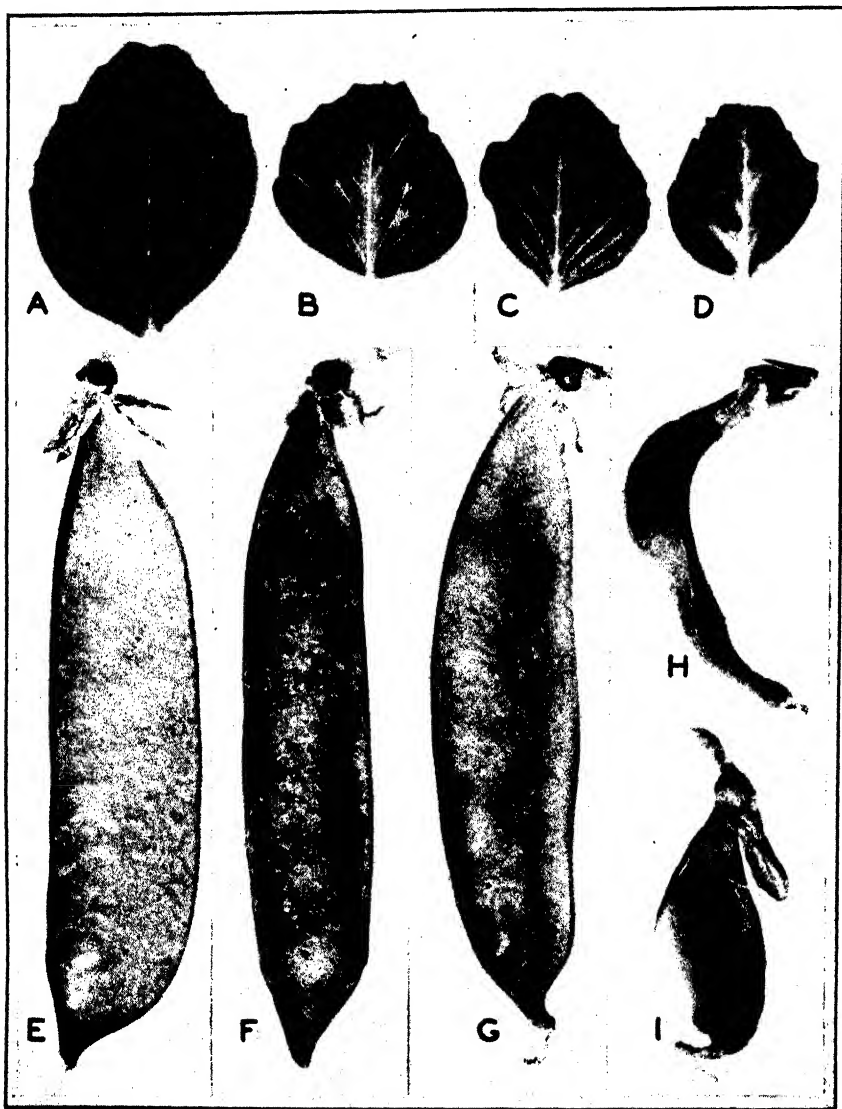


FIGURE 3.—Symptoms on leaflets and pods of peas inoculated with strain 14: A, Leaflet from uninoculated Wisconsin Perfection plant; B, leaflet from Alderman inoculated with strain 14 (note diffuse mottle); C, D, leaflets from Wisconsin Perfection inoculated with yellow strain of 14 (mottle is more distinct than in B); E, pod from uninoculated Alderman; F, G, necrosis of pods from field-inoculated Alderman plants; H, I, twisting and stunting of younger pods from the same variety.

these shoots (fig. 5) was due to extreme shortening of the internodes and distortion of the leaves. Mottling of leaves and necrosis of stems were less common on secondary branches, but necrotic flecking of leaves and stems usually developed. Blossoms which were well



FIGURE 4.—Wisconsin Perfection pea plant (A) 3 weeks after inoculation with strain 14. Note extreme reduction in size as compared with uninoculated plant (B), as well as necrosis of lowest leaves and mottle of young leaflets.

developed at the time of initial symptoms usually dropped, while small pods became distorted and necrotic (fig. 3, *H*, *I*). Some older pods remained quite normal, while others showed severe necrosis (fig. 3, *F*, *G*), and peas from the latter were occasionally mottled or necrotic. On the secondary branches only a few pods set, and these



**FIGURE 5.**—Portion of an Alderman plant naturally infected in the field with strain 14. The internodes of side branches remain short, and faint necrosis occurs along the veins of some leaflets.

either developed normally or showed symptoms as described above. The virus was readily recovered from stem, leaves, and pods.

#### STRAIN 17

The symptoms of this virus closely resemble those described above for strain 14 on the same three pea varieties. Stem necrosis, however, is more pronounced and vein clearing of the young leaves is more common than mottling. Stunting is equally severe, and plants showing necrosis usually die prematurely.

#### CUCUMBER VIRUS 1

In no case has this virus been recovered from inoculated pea plants, although a few such plants have shown definite symptoms. In a 10-plant series of Alderman, 1 plant developed a necrosis of rubbed leaflets and petioles; in a few days necrosis had advanced 1 cm. along the stem. Besides this necrosis, stunting was the only additional symptom noted on this plant. In another plant in the same series distinct stem and top necrosis occurred, followed by early death of the plant. In still another series of this variety faint necrotic streaking developed on stems of 3 plants. Out of 12 inoculated plants of the Alaska variety, one showed stem necrosis at the base of an inoculated leaf. In 3 separate series inoculation to Wisconsin Perfection gave negative results. From these experiments it is doubtful if pea should be considered a host of the strain of cucumber virus 1 used, but it is indicated that the virus may occasionally enter and progress within the tissue.

#### CELERY VIRUS 1

Similar preliminary results indicated that this virus may also go to peas. In one series of 10 Alderman plants necrosis of inoculated leaflets and stipules developed on 2 without the appearance of systemic symptoms. In another 4-plant series of Alaska speckle necrosis of leaflets and stipules occurred on 3 of the plants. No attempt was made to recover the virus. In 3 separate series inoculation to Wisconsin Perfection failed to produce symptoms. Further studies are needed to determine whether peas may be considered a host of this virus.

#### SUBSTRAINS OF VIRUS 14

Tobacco leaves infected with strain 14 occasionally showed bright yellow areas on the older leaves (fig. 6, A) similar to those described by McKinney (20) for common tobacco mosaic and by Price (24) for cucumber mosaic; from these areas "yellow" substrains were isolated (fig. 6, B, C). In addition, two other substrains, "dark green" and "normal green," were isolated from tobacco leaves infected with strain 14. The "dark green" isolate was obtained from a small raised area of tissue darker than normal green. The other was from a nearly normal green area on the same leaf.

On tobacco the yellow substrains produced amounts of chlorosis varying with the isolate (fig. 6, B, C). Mottle was accompanied by much leaf distortion and stunting. One of these isolates (fig. 6, B) was used to inoculate cucumber, Wisconsin Refugee bean, Wisconsin Perfection pea, and Black cowpea (*Vigna sinensis* Endl.) in the green-

house. On cucumber the small circular chlorotic primary lesions rapidly spread to produce a yellowing and quick death of the cotyledons. Chlorotic sunken streaks next appeared on the hypocotyl.



FIGURE 6.—A, Bright yellow spot in a tobacco leaf infected with strain 14. From the tissue of such spots "yellow" strains were isolated. B, C, Leaves from tobacco plants infected each with a different "yellow" isolate of strain 14.

followed by a yellowing of the true leaves. Infected plants usually died soon after systemic symptoms appeared. Initial symptoms on Wisconsin Refugee bean and Black cowpea were epinasty of the rubbed unifoliate leaves followed by chlorotic areas on these leaves.

Systemic symptoms frequently developed as a severe necrosis of stems, petioles, and leaves. Necrosis of the pulvinus was especially prominent. Most of these plants died prematurely. On those which survived or did not show necrosis a bright yellow mottle of the leaves and severe stunting of the plants developed. The mottle symptoms on cowpea were similar to those described by Price (24) for his  $Y_1$  and  $Y_2$  strains. Wisconsin Perfection peas infected with the yellow substrains show more distinct yellow mottle (fig. 3, *C* and *D*), more leaf distortion, severe necrosis, stunting, and a higher mortality than do those infected with the original strain.

The dark green substrain was also compared directly with the other substrains. On tobacco it showed a mild but distinct mottle of dark green raised areas dispersed on a normal green background, and distortion and stunting were less severe. In general these symptoms were also typical of the dark green isolate on cucumber, Black cowpea, Wisconsin Refugee bean, and Wisconsin Perfection pea. The normal green substrain produced symptoms on the above hosts identical with those of the original strain. In all cases the yellow substrain produced the most severe symptoms, while the dark green strain was milder than any of the substrains or the original isolate.

From these experiments it is evident that, as has been reported for the cucumber mosaic virus (24), it is possible to isolate a number of substrains which remain stable, give distinct symptoms, and differ from one another in their severity on the respective hosts.

#### RELATION OF TEMPERATURE TO SYMPTOM EXPRESSION

Idaho Refugee, Kentucky Wonder Pole (regular), and Full Measure beans, and Alderman and Wisconsin Perfection peas were included in a study of the relation of temperature to expression of symptoms. After inoculation with strain 14 by the usual method, 10-plant groups of each variety were placed in temperature-controlled greenhouses at 16°, 20°, 24°, and 28° C. An equal number of check plants was placed at each temperature.

Epinasty of the unifoliate leaves was the initial symptom on bean at all temperatures, but time of appearance varied from 2 to 3 days after inoculation on plants at 28° C. to a week or more at 16°. Diffuse yellow areas next developed on the unifoliate leaves of plants at 28°, and in 5 days vein clearing and distinct mottle appeared; at the end of 2 weeks severe necrosis of stems, leaves, and growing tip had developed on 20 percent of the plants. The progress of the disease in plants at 24° was somewhat slower, and at 2 weeks vein clearing, mottle, and stunting, but no necrosis, were evident. The mottle developed still more slowly at 20°, but at 2 weeks the disease had advanced nearly as far as at 24°. At the end of the experiment the only symptom on plants at 16° was epinasty of the inoculated primary leaves.

Infected Wisconsin Perfection pea plants first showed mottling of the leaves at 24° C. in about 11 days, and stunting of the inoculated plants at all temperatures was then evident. In one experiment, 18 days after inoculation, 9 of the 10 plants at 28° were either dead or severely wilted and a few showed a faint necrosis of the stem; at 24° 4 of the 10 plants had developed faint necrosis of the stem and had died prematurely; at 20° typical mottling developed on 6, and on 1

necrosis of the growing tip occurred; at 16° none of the plants showed symptoms other than stunting.

Although the disease is most severe in its effect on peas and beans at 28°, the most typical symptoms develop at 24°. At 20° they occur more slowly and with less severity, although they are nevertheless typical in appearance. Symptoms other than epinasty are completely masked at 16° C., but the virus is readily recovered from inoculated plants held at this temperature.

#### PROPERTIES OF THE VIRUSES

The properties of strains 14 and 17 were studied to determine how closely they conform to those reported by Johnson (15), Hoggan (10), and Price (24) for cucumber mosaic viruses and by Wellman (33) for celery virus 1. In the matter of longevity in vitro cucumber virus 1 used in other studies reported herein was compared with strains 14 and 17.

Expressed juice from tobacco plants recently infected with the viruses was treated and then used to inoculate tobacco as the test plant. Ten plants were used as a test unit and each experiment was repeated at least once (table 2).

TABLE 2.—Comparison of certain properties of cucumber virus 1, strain 14, and strain 17 as determined by inoculation of tobacco<sup>1</sup>

Thermal inactivation point					Longevity in vitro								Tolerance to dilution							
Temperature (° C.)	Strain 14		Strain 17		Time aged (days)	Strain 14		Strain 17		Cucum-ber virus 1		Dilution	Strain 14		Strain 17					
	Trial		Trial			Trial		Trial		Trial			Trial		Trial					
	1	2	1	2		1	2	1	2	1	2		1	2	1	2				
Untreated.....	10	10	10	10	None	10	9	10	10	10	10	None	10	5	9	9				
45°.....	7	9	10	10	1	10	9	9	9	9	9	1-10	10	6	7	4				
50°.....	6	9	10	10	2	10	9	9	9	9	9	1-100	9	2	6	2				
55°.....	5	5	7	7	3	9	4	4	4	4	4	1-1,000	2	1	4	1				
60°.....	10	3	3	1	4	9	5	2	8	10	4	1-10,000	1	0	0	0				
65°.....	5	0	1	0	5	10	3	4	6	4	6									
70°.....	0	0	1	0	6	9	1	0	6	1	6									
75°.....	0	0	0	0	7	10	1	0	1	0	1									
					8	6	0	0	0	0	0									
					9	0	0	0	0	0	0									
					10	1	0	0	0	0	0									

<sup>1</sup> Figures represent the number of tobacco plants infected out of 10 inoculated with the virus indicated.

#### THERMAL INACTIVATION POINT

The method used for heating the virus samples was the same as that described by Price (23) and later used by him in comparing yellow strains of cucumber mosaic (24). The thermal inactivation points of strains 14 and 17 were found to be between 65° and 70° C., when exposed at this temperature for 10 minutes. These figures are in close agreement with those reported by Hoggan (10) for cucumber mosaic viruses and are within the range given by Johnson (15) for classification of the cucumber virus group. Price (24), using the same method, obtained a variation from 64° to 72° for eight different strains, but concluded that they were essentially similar in their resistance to heating. Wellman (33) found that celery virus 1 occasionally

remained infective after treatment at 75°, but that it was inactivated in all samples treated at 80°.

#### LONGEVITY IN VITRO

The expressed juice from aging studies was stored in stoppered test tubes at 20° to 22° C. The longevity in vitro was 7 to 8 days for strains 14 and 17. In one trial, however, one plant became infected by inoculum aged for 10 days, although none were diseased after inoculation with the same extract at 9 days. These results are in general accord with those for cucumber virus 1, which was found to lose its infectivity at 6 to 7 days, though Johnson (15) and Hoggan (10) report 3 and 5 days, respectively, for this virus.

#### TOLERANCE TO DILUTION

Dilutions were made with sterile distilled water and the plants were inoculated immediately. There was no significant difference between strains 14 and 17. The maximum dilution at which infection occurred was usually 1-1,000, although in one case it was 1-10,000. These results are in general agreement with those reported by other workers for the cucumber mosaic virus group (10, 33), which ranges from 1-1,000 to 1-100,000 in maximum dilution from which infection may be secured.

#### INSECT TRANSMISSION

The green peach aphid, *Myzus persicae* Sulz., was studied as a possible vector of strains 14 and 17. Nonviruliferous aphids were raised on healthy cabbage plants and later transferred to a variety of infected hosts, on which they were allowed to feed from 48 to 72 hours. At the end of this period 10 to 15 aphids were transferred to each healthy plant to be infected. After another feeding period of 48 to 60 hours the aphids were killed by fumigation. In the transfer of a small number of insects a brush of red sable's hair was used, but for larger numbers the leaf method of Hoggan (8) was found useful.

Strain 14 was transferred by means of aphids from tobacco to tobacco, yellow sweetclover (*Melilotus officinalis* (L.) Lam.) to pepper (*Capsicum annuum* L. var. California Wonder), kidney bean to pepper, spinach (*Spinacia oleracea* L. var. Bloomsdale) to pepper, pepper to pepper, and pea to jimsonweed (*Datura stramonium* L.). Strain 17 was also transmitted by the same vector from pea to spinach and from spinach to spinach. Six plants served as a test unit in each case. The virus was recovered from all hosts except jimsonweed. The incubation period in the hosts varied from 14 to 21 days. No experiments were conducted to demonstrate the length of time required for the aphid to become infected or the length of time it remained infective.

#### SEED TRANSMISSION

Transmission of bean virus 1 through the seed of common bean was first demonstrated by Reddick and Stewart (29), but bean virus 2 was found nontransmissible by Pierce (21). Doolittle and Gilbert (5) have given evidence to prove that cucumber mosaic may be transmitted through the seed of the wild cucumber (*Micrampelis lobata* (Michx.) Greene), and Kendrick (16) demonstrated occasional transmission of the same virus through the seed of muskmelon varieties



Honey Dew and Persian (*Cucumis melo* L. vars. *inodorus* and *reticulatus* Naud.).

In a total of 580 pea and 440 bean seedlings grown from seed of plants infected with strain 14 none was found to be affected with the virus. The pea and bean seeds used were obtained from both naturally and artificially infected field plantings reported earlier in this paper. Further trials are needed to determine conclusively whether this virus is transmitted through pea and bean seed, but these preliminary trials suggest that it is not. No seed transmission studies with strain 17 have been made.

#### HOST RANGE

Rather than ascertain the infectivity of strains 14 and 17 on a series of plant species chosen at random, it was considered more to the point to make a study on known hosts of the cucumber mosaic group of viruses. This list includes those plants most commonly infected in nature with cucumber virus 1 and, secondly, those which have been more recently reported as hosts of this virus or strains closely related to it. As strains 14 and 17 were first observed on peas and beans, other legume hosts, such as lima bean, cowpea, lupine (*Lupinus angustifolius* L. and *L. hartwegii* Lindl.), and sweetclover were of special interest. A list of the plant species susceptible to one or more of the four viruses studied in this paper and the cardinal symptoms produced on each host are given in table 3. This list includes 19 species belonging to 16 genera and 6 families. In addition none of the four viruses was found to be infectious on White Dutch Clover (*Trifolium repens* L.) or soybean (*Soja max* (L.) Piper var. Manchu). From 5 to 50 plants or more of each species were inoculated, and with few exceptions the virus was recovered when signs of disease developed.

A study of table 3 will reveal that all plants susceptible to cucumber virus 1 are also hosts of strain 14, and, in addition, the latter virus brought about infection on Fordhook Mammoth Pod lima bean and yellow sweetclover. Broadbean, susceptible to celery virus 1, was the only plant tested not infected by strain 14. The celery virus also produced infection on all plants tested except Fordhook Mammoth Pod lima bean and yellow sweetclover. The host range of strain 17 paralleled that of cucumber virus 1 and in addition included yellow sweetclover.

It is not the purpose of this paper to describe in detail the symptoms on all the hosts infected with different viruses. However, it should be mentioned that in general the symptoms on the respective hosts were similar as to type, and any variations between viruses were mostly in degree or intensity. For instance, under field conditions, cucumber and squash infected with strain 14 not only produced plant symptoms identical with those of cucumber virus 1, but also gave rise to wartlike areas and distortion of the fruit. The similarity of the mottle symptoms of the four viruses may be seen in figure 10, while typical variations in these symptoms are shown on tobacco in figure 7. It is at once apparent that symptoms produced by strain 17 on this host are much milder than those of the other two viruses. Symptoms of strain 17 (fig. 7, C) most closely resemble those of "cucumber mild mosaic virus" described by Hoggan (10).

Strain 17 was somewhat unstable and occasionally symptoms similar to those of cucumber virus 1 appeared on tobacco. Transfer of the virus from such plants give rise to stable substrains. Strain 14 produced many types of symptoms on tobacco leaves, including oak-leaf patterns, savoy, and reduction of leaf lamina. The principal difference in this regard between strain 14 and cucumber virus 1 was a slightly greater amount of yellow in the lighter areas of the mottle pattern produced by the former.

On cowpea, primary lesions were produced by all four viruses (fig. 13, *A*) similar to those described by Price (24). The necrotic lesions produced by cucumber virus 1 were slightly larger and more numerous than those of the other three viruses. In addition, strain 14 produced other primary symptoms, such as circular chlorotic areas which later often became partly necrotic (fig. 13, *B*, *C*) and had a tendency to spread out along the main veins. Systemic symptoms, such as mottling, leaf distortion, bronzing (fig. 13, *D*), and severe stunting, developed on this host (fig. 14, *B*).

Dark necrotic lesions were produced on the inoculated primary and true leaves of watermelon by cucumber virus 1 and strains 14 and 17 not unlike those described by Wellman (35) for celery virus 1. The yellow halo about the necrotic lesions was usually conspicuous and on one plant inoculated with strain 14 a yellowing developed over the entire plant. An attempt to recover the virus was unsuccessful.

The infection of Henderson Bush lima by all four viruses is of interest in view of Harter's (7) report that a strain of cucumber mosaic was infectious to eight varieties of small-seeded limas but not to the large-seeded Fordhook types tested. Of the writers' four viruses only strain 14 infected Fordhook Mammoth Pod. The symptoms of the four viruses on Henderson Bush lima were similar and developed as epinasty, vein clearing, mottling, stunting, and occasionally bronzelike necrosis of leaves and necrosis of stems and growing tip. Strain 14 produced the most severe symptoms of the four viruses and death of the plants was common. The symptoms observed by the writers on this host are not unlike those described by Harter (7) except that the latter did not mention any necrotic condition. Since cucumber virus 1, as well as the other three strains used in this study, infects Henderson Bush lima bean, it is obvious that this is not a good differential host for strains of the cucumber virus group.

The serious disease that developed from inoculating lupine with strains 14 and 17 and cucumber virus 1 is similar to the lupine disease reported from Germany by Köhler (17, 18), who considered it to be identical with Ainsworth's (1) "yellow-mottle mosaic" (cucumber virus 1 of Johnson).

Wellman (33) reported local lesions on leaves of broadbean inoculated with celery virus 1 but did not obtain systemic infection. In the course of the experiments reported herein typical local lesions were also obtained with this virus, and in a few cases systemic symptoms also developed.

Inoculations of strain 14 to perennial chrysanthemum gave symptoms which suggest a similarity to those of other viruses on this plant as reported by Valteau (31) and Burnett (2), although the reaction of the viruses described by these investigators was different on other hosts from that of the four studied here.

TABLE 3.—“Type symptoms” as produced on various hosts by cucumber virus 1, together with variations from “type symptoms” produced by celery virus 1, strain 14, and strain 17

Species and common name	Type symptoms <sup>1</sup> (cucumber virus 1)	Variations from type symptoms as described for cucumber virus 1		
		Celery virus 1	Strain 14	Strain 17
<i>Nicotiana tabacum</i> L. (tobacco—var. Havana 38).	Local: Occasional diffuse chlorotic areas in 7 to 9 days. Systemic: Vein clearing in 7 to 10 days; mottling, distortion, stunting (fig. 7, B). Local: Usually chlorotic areas. Systemic: Similar to symptoms on tobacco.	Local: Small circular, bleached areas. Systemic: Similar (to type symptoms). <sup>1</sup> Local: Similar to symptoms on Havana tobacco. Systemic: Similar.	Local: Usually chlorotic areas. Systemic: Similar, but with more yellow mottle (fig. 7, A). Local: Faint chlorotic areas. Systemic: Similar; also netlike mottle and more puckering.	Local: Same as strain 14. Systemic: Faint mottle, irregular surface; no distortion or stunting (fig. 7, C). Local: Same (as type symptoms). <sup>1</sup> Systemic: Netlike mottle remaining diffuse; no distortion or stunting. Systemic: Indistinct, reticulate mottle; later symptoms often masked. Systemic: Diffuse mottle; dark green, circular patterns. Systemic: No stunting or distortion.
<i>Nicotiana glauca</i> L.				
<i>Lycopersicon esculentum</i> Mill. (tomato—var. John Baer).	Systemic: Vein clearing, mottle, distortion, stunting, filiform leaves. Local: Chlorotic areas (3 to 20 mm. diameter); later necrotic or bleached. Systemic: Mottling, distortion, stunting.	Systemic: Mild mottle; leaf distortion uncommon. Local: More diffuse than type symptoms. Systemic: Similar to strain 14.	Local: Same. Systemic: Vein clearing, dark green mottle; distortion, stunting (fig. 9, D). Systemic: Similar to type; also dark green islands; yellowing, necrosis (fig. 9, C). Local: Similar to celery virus 1, but later enlarge. Systemic: Similar; also chlorotic streaking on stems (fig. 10, D). Systemic: Also chlorotic blotch-like areas; necrosis of older leaves (fig. 11, B).	Local: Similar to strain 14, but less necrosis. Systemic: Similar to but more severe than strain 14 (fig. 10, B). Systemic: Yellow mottling, circular chlorotic areas; necrosis of leaf margins.
<i>Datura stramonium</i> L. (Jimson-weed).				
<i>Capsicum annuum</i> L. (pepper—var. California Wonder).				
<i>Cucumis sativus</i> L. (cucumber—var. White Spine).	Local: Occasional diffuse chlorotic areas on cotyledons. Systemic: Vein clearing, mottle, cupping, dark green areas, puckering, stunting (fig. 10, C). Systemic: Mottling, puckering, stunting (fig. 11, A).	Local: Chlorotic areas 3 to 4 mm. diameter; in 3 to 7 days necrotic. Systemic: Similar (fig. 10, A). Systemic: Same.		
<i>Cucumis melo</i> L.—vars. <i>reticulatus</i> Naud. and <i>inodorus</i> Naud. (muskmelon—vars. Rocky Ford and Honey Dew, respectively).	Local: Chlorotic areas on cotyledons (3 to 5 mm. diameter); few later became necrotic. Systemic: Vein clearing; first chlorotic blotches; later mottling, stunting, savoy, mottling, and distortion of fruit.			
<i>Cucurbita pepo</i> L. (squash—vars. Giant Summer Crookneck and Cocozelle).	Local: Chlorotic lesions on cotyledons later necrotic, spreading (fig. 12, C); similar lesions also on true leaves with yellow halo. Systemic: None.	Local: Similar. Systemic: Similar (Wellman (35)). Local: Black necrotic local lesion. Systemic: Vein clearing, mild mottle, stunting (Wellman (35)).	Local: Similar (see fig. 12, D, E, F). Systemic: Less savoy and distortion (fig. 11, C). Local: Lesions smaller, less necrotic spreading (fig. 12, B).	Local: Same. Systemic: Milder, more diffuse, blotchy type mottle; less stunting and distortion (fig. 11, D). Local: Same as for strain 14 (fig. 12, A).

<i>Phaseolus lanatus</i> L. (lima bean—var. Henderson Bush).	Local: Diffuse chlorotic areas on unifoliates, later brick-red necrosis spreading along veins. Systemic: Vein clearing, mild yellow mottle; necrosis of young leaves and growing tip; stunting. No infection.	Local: Same. Systemic: Same.	Local: Similar. Systemic: Slightly more severe.	Local: Occasional small brick-red necrotic lesion. Systemic: Diffuse mottle; bronzing of leaves; necrosis of stem.
<i>Phaseolus lanatus</i> L. (lima bean—var. Fordhook Mammoth Pod).	No infection.	No infection.	No infection.	No infection.
<i>Vigna sinensis</i> Endl. (cowpea—var. Black).	Local: Dark-red necrotic lesions on unifoliolate leaves (2 to 3 mm. diameter) in 2 to 3 days (figs. 13, 14).	Local: Similar.	Local: Similar. Systemic: More yellowing, severe stunting (figs. 15, 16).	Local: Few necrotic lesions.
<i>Lupinus angustifolius</i> L. and <i>L. hartwegii</i> Lindl.	Local: Occasional bronze-like necrotic areas. Systemic: Yellowing, faint mottle, necrotic flecking, drooping of leaves, stunting, stem necrosis, death (figs. 15, 16).	Not tested.	Local: Similar. Systemic: More yellowing, severe stunting (figs. 15, 16).	Systemic: Similar to but milder than strain 14.
<i>Melilotus officinalis</i> (L.) Lam. (yellow sweetclover).	No infection.	No infection.	Systemic: Vein clearing, mild mottling, yellowing, necrotic flecking and drooping of leaves; stunting. No infection.	Systemic: Similar to strain 14, but less stunting and often no symptoms.
<i>Vicia faba</i> L. (broadbean).	do	Local: Dark brown lesions in 8 days. Systemic: Yellowing or necrosis of leaves and stems; stunting; death. Local: Same as for strain 14. Systemic: Same as type symptoms.	No infection.	No infection.
<i>Stinacia oleracea</i> L. (spinach—var. Bloomsdale).	Systemic: Mild mottle, yellowing, severe puckering, malformation, and stunting. Systemic: Mild mottle, filiformity, raised blisterlike areas, stunting.	Local: Same as for strain 14. Systemic: Same as type symptoms.	Local: Diffuse chlorotic areas. Systemic: Similar to type symptoms (figs. 9, 10).	Systemic: Fine mottle, yellowing and irregular leaf surface.
<i>Apium graveolens</i> L.—var. <i>dulce</i> DC. (celery—var. Golden Self-blanching).	Systemic: Mild mottle, filiformity, raised blisterlike areas, stunting.	Systemic: More brilliant yellow mottle, curvature of petiole.	Systemic: Symptoms more mild.	Systemic: Very mild mottle.
<i>Zinnia elegans</i> Jacq. (Zinnia—var. Liliput).	Systemic: Vein clearing, yellowing, mottling, and stunting. Not tested.	Systemic: More severe mottle, distortion, chlorosis. Not tested.	Systemic: Same.	Not tested.
<i>Chrysanthemum morifolium</i> Ram. (perennial chrysanthemum).	Not tested.	Not tested.	Systemic: Yellowing, mild mottle, stunting.	Do.

<sup>1</sup> Symptoms produced on the various hosts by cucumber virus 1 are designated as "type symptoms." Those produced by celery virus 1, strain 14, and strain 17 on the respective hosts are not described in detail unless they differ significantly from the "type symptoms." Symptoms are treated in 2 groups, local and systemic; when one or the other of these is omitted it is implied that they did not occur; when one or the other occurs but is similar to the "type" or the same as the "type," the fact is so stated.

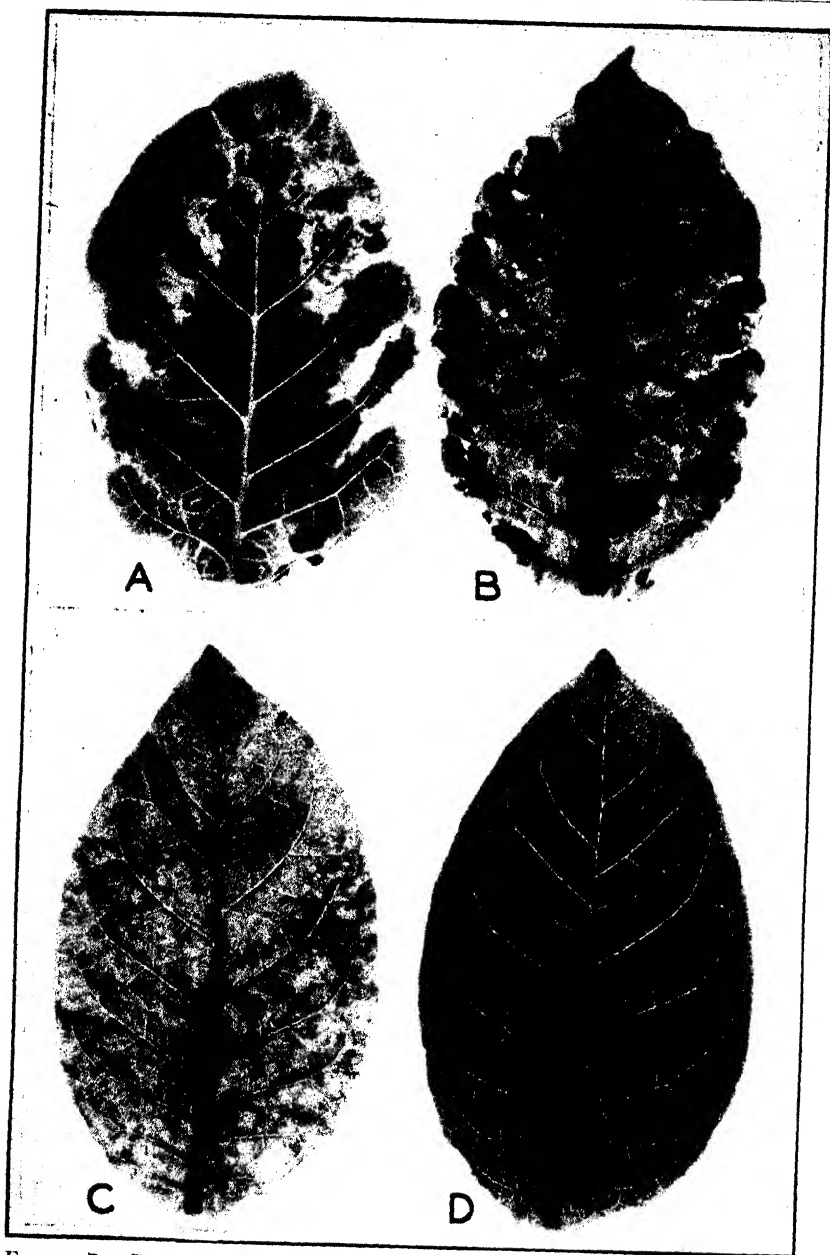


FIGURE 7.—Comparable leaves from tobacco plants inoculated with: *A*, Strain 14; *B*, cucumber virus 1; *C*, strain 17 (note the diffuse mottle); *D*, leaf from an uninoculated plant in the same series.

## SPECIFIC IMMUNITY PRODUCED IN ZINNIA BY STRAIN 14

Price (25) has shown that zinnia plants mottled by any of several different strains of cucumber mosaic virus are immune from his strain



FIGURE 8.—A, Leaf from uninoculated tomato; B, leaf from tomato inoculated with strain 14 (note stunting and shoe-string type of leaflet distortion; a diffuse mottle also occurs).

6, which produces necrotic primary lesions. Preliminary tests indicate that zinnia leaves mottled by the writers' cucumber mosaic virus strain 14 are also afforded protection from infection by strain

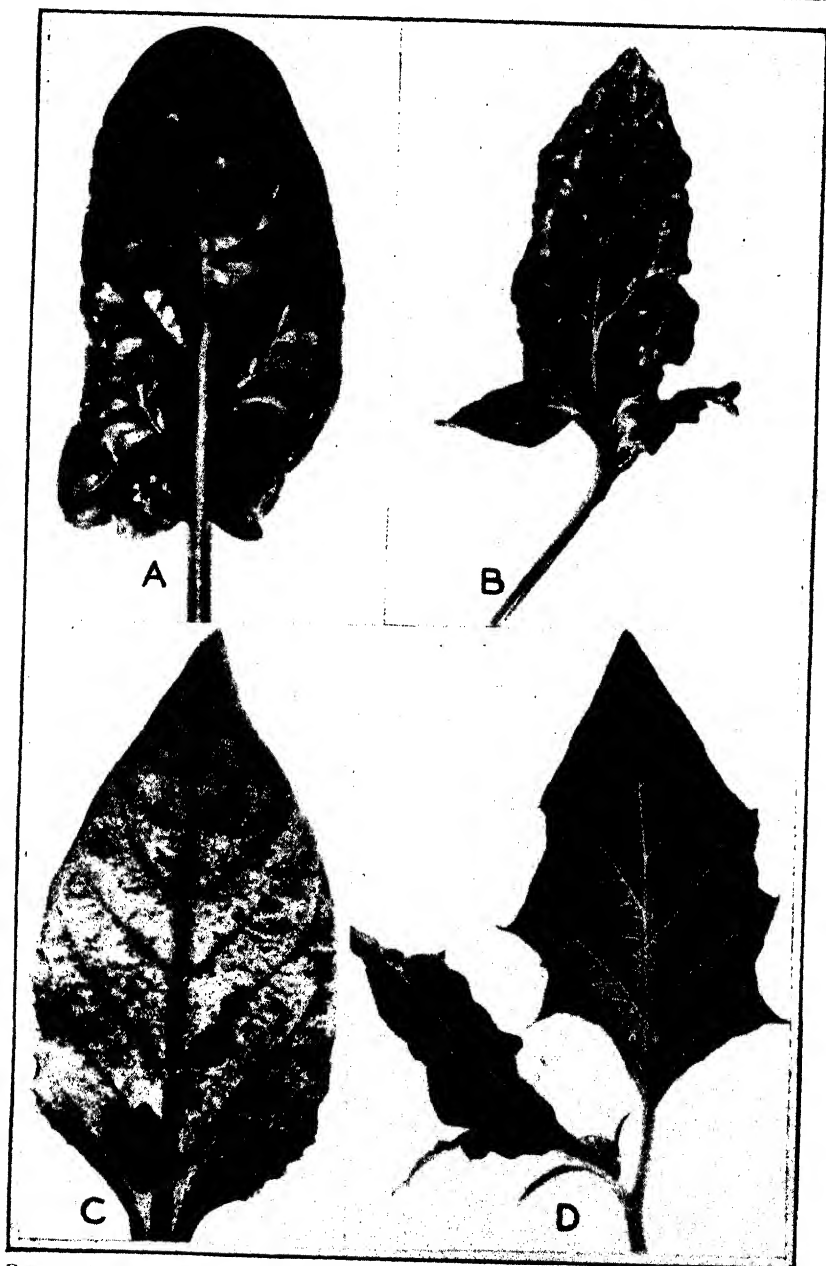


FIGURE 9.— *A*, Leaf from a healthy spinach plant; *B*, leaf from a spinach plant infected with strain 14 (note mottle, distortion, and stunting); *C*, leaf of California Wonder pepper infected with strain 14, showing mottling and dark green island; *D*, diffuse mottle and distortion in leaflets of *Datura stramonium* infected with strain 14.

6.<sup>5</sup> In one test 5 young zinnia plants were inoculated with strain 14 and allowed to become thoroughly mottled; then 4 leaves on each plant were inoculated with strain 6. Five healthy plants were inoculated with the latter virus only, and set apart for controls.

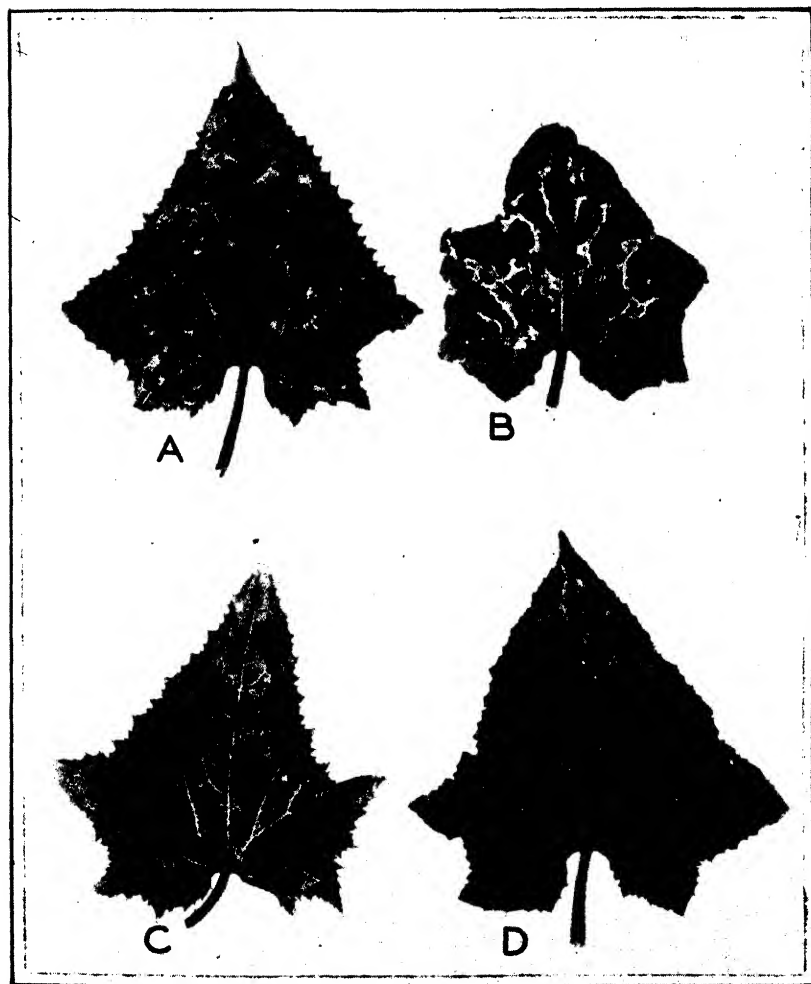


FIGURE 10.—Comparable leaves from White Spine cucumber plants inoculated with: A, Celery virus 1; B, strain 17; C, cucumber virus 1; D, strain 14 (note the similarity in the mottle symptoms on this host).

Lesions did not develop in any of the mottled leaves. The 5 control plants developed a total of 173 lesions. In a second test new leaves on each of the same 5 plants infected with strain 14 were inoculated with Price's strain 6. As in the first trial no lesions developed on the leaves mottled by strain 14, while 5 new control plants developed a

<sup>5</sup> A sample of cucumber mosaic virus strain 6 was kindly furnished by Dr. W. C. Price.



total of 129 lesions. A third series of 5 plants inoculated with strain 14 were again inoculated with strain 6 before all leaves showed a distinct mottle. Four lesions developed on 2 of the leaves that did not show mottling. Five control plants inoculated with strain 6 only, developed a total of 81 lesions on the same number of leaves.

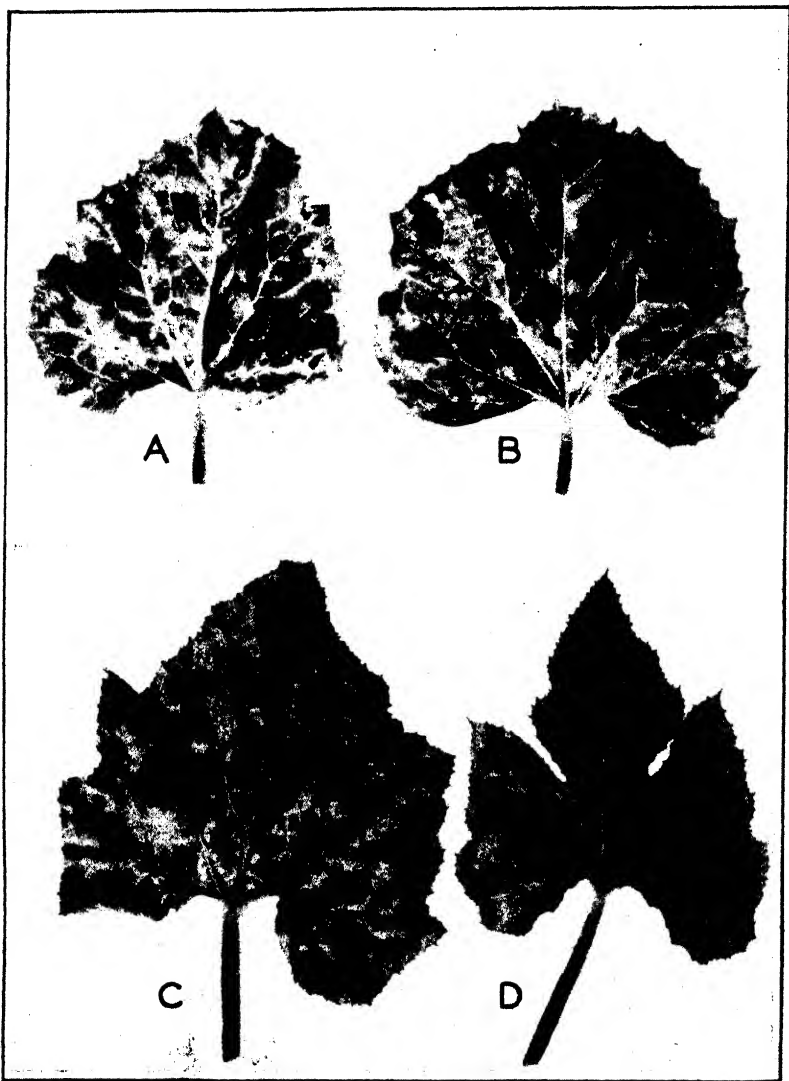


FIGURE 11.—A, Leaf of Honey Dew muskmelon plant inoculated with cucumber virus 1; B, leaf of same variety as in A inoculated with strain 14 (note the similarity in mottle symptoms); C, leaf of Cocoselle squash plant inoculated with strain 14; D, leaf of same variety as in C inoculated with strain 17 (note the more diffuse mottle in the latter).

Five zinnia plants mottled with cucumber virus 1 were also inoculated with strain 6, but no lesions developed on any of the leaves.

These preliminary tests tend to confirm Price's (25) report that zinnia plants mottled with cucumber mosaic virus are immune from infection by a second strain of the same virus. If the failure of

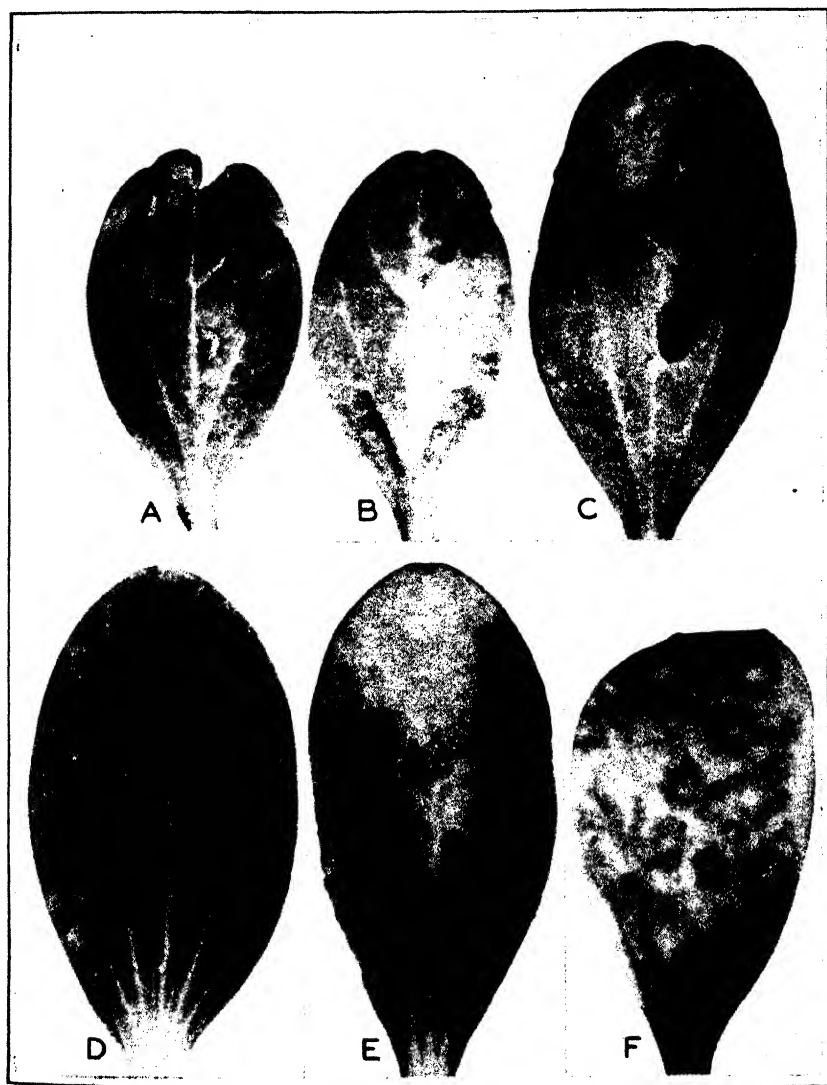


FIGURE 12.—A, Cotyledon of Kleekey Sweet watermelon inoculated with strain 17 (note the typical necrotic local lesions and their similarity to those on cotyledons from plants inoculated with strain 14 (B) and cucumber virus 1 (C)). D, E, F, Cotyledons of Cocozelle squash inoculated with strain 14: Early chlorotic lesions are shown in D; necrosis sometimes follows, as in E; commonly the early chlorotic lesions acquire a dark green halo, while the remainder of the cotyledon turns yellow, as in F.

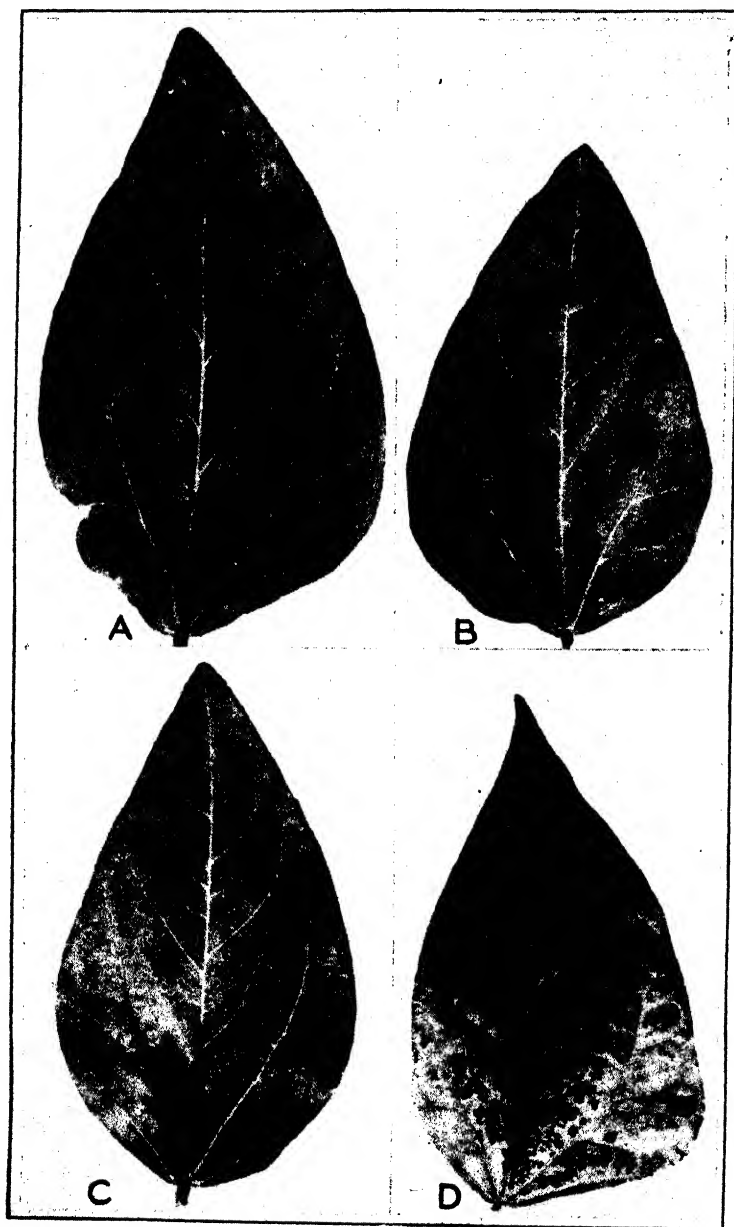


FIGURE 13.—A, Necrotic lesions on inoculated unifoliate leaf of Black cowpea infected with cucumber virus 1, (celery virus 1, strain 14, and strain 17 produce similar but fewer lesions on this host); B, larger chlorotic local lesions which have become necrotic, a type which also follows inoculation with strain 14; C, later stage of type shown in B, necrosis having spread along the veins; D, bronzing and yellowing on leaflet of a first trifoliate from a Black cowpea plant infected systemically with strain 14.



FIGURE 14.—Black cowpea: A, Leaf from uninoculated control; B, extreme mottle and distortion on leaf from plant systemically infected with strain 14.

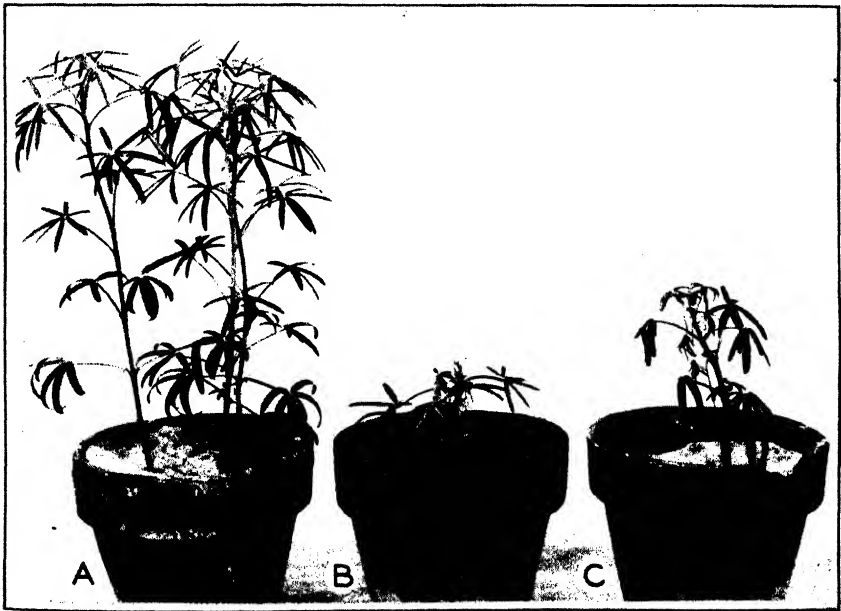


FIGURE 15.—A, Uninoculated plants of *Lupinus angustifolius* L.; B, severe stunting of plant infected with strain 14 (mottling, chlorosis, wilting, and necrosis also occur); C, comparable plant infected with cucumber virus 1.

necrotic lesions to develop on the leaves mottled with either cucumber virus 1 or strain 14 is due to a specific acquired immunity it would seem to demonstrate further the close relationship of strain 14 to the cucumber mosaic group.

#### SEPARATION FROM ONE ANOTHER OF THE FOUR VIRUSES STUDIED

Strains 14 and 17 may be separated from mixtures with bean virus 1 and bean virus 2 (21), pea virus 1 and pea viruses 2A, 2B, and 2C (30) by inoculation to either tobacco or tomato. The five last-named viruses may also be freed from strains 14 and 17 by transfer to broadbean.

The four viruses studied cannot be completely separated from one another when present in admixture. However, certain combinations of these viruses in the same plant may be readily separated out by means of differential hosts. Strain 14 can be isolated from a mixture of any of the other viruses by inoculation to kidney bean, cowpea, or Fordhook Mammoth Pod lima bean. Strain 17 can be separated from either cucumber virus 1 or celery virus 1 by inoculation to garden pea or yellow sweetclover, but it cannot be separated from a mixture with strain 14. Celery virus 1 is freed readily from the other three viruses by inoculation to broadbean. Cucumber virus 1 cannot be separated from any of the other viruses by the methods employed.

#### DISCUSSION

In this investigation an attempt has been made to describe and identify two viruses which were found occurring naturally on pea and bean. On the basis of symptoms, host range, modes of transmission, physical properties, and immunity studies it appears that they are related to the cucumber mosaic virus group, of which cucumber virus 1 may be given as the type virus (4, 15). Since the term "cucumber mosaic virus group" has been commonly used by a number of workers in classifying a particular strain or group of related viruses, the following review of the more pertinent literature is given in an effort to orientate the use of this term and to focus attention on the fact that strains apparently isolated experimentally from the same well-recognized virus may differ in symptoms and hosts affected as do those isolated from natural material.

In 1916, Doolittle (3) and Jagger (11), working independently, reported the occurrence of a transmissible mosaic disease of cucumber. Later Doolittle (4) gave a more complete description of symptoms, host range, physical properties, insect and seed transmission of his virus. He found that expressed juice from mosaic plants was rendered noninfectious when heated for 10 minutes at 70° C. or aged in vitro more than 3 to 5 days, and that dilutions of 1 to 10,000 still produced infection. All species of Cucurbitaceae tested by Doolittle and Walker (6) were found susceptible except those of the genus *Citrullus* (watermelon and citron). Of the latter only the green-seeded citron was infected. Additional hosts in other families were found susceptible, including tobacco, pepper, *Physalis* spp., catnip (*Nepeta cataria* L.), *Martynia louisiana* Mill., milkweed (*Asclepias syriaca* L.), pokeweed (*Phytolacca decandra* L.), and pigweed (*Amaranthus retroflexus* L.). Johnson (15) suggested cucumber virus 1 as the

technical name for the cucumber mosaic virus and recommended that the one described by Doolittle (4) be regarded as the type virus.

The natural occurrence of a number of cucumber mosaic viruses which differ either in symptoms, physical properties, or host range have been reported and some have been isolated experimentally. Jagger (12) distinguished between "white pickle" mosaic and a second mosaic of cucumber which caused a mottle of leaves but gave rise to no symptoms on the fruit. He (13) also reported a third mosaic disease on summer crookneck squash and pie pumpkin which could not be transmitted to cucumber. E. M. Johnson (14) on the basis of symptoms produced on tobacco described the following three types of cucumber mosaic. Valleau and Johnson (32) had earlier characterized type 1 by the term "puffed;" this type produced symptoms on cucumber similar to those described by Doolittle (4). Cucumber mosaic types 2 and 3 produced different symptoms on tobacco but were similar on cucumber and certain other hosts. Porter (22) made a study of cucumber mosaic obtained from a number of States, including Iowa, New York, California, Kansas, and Kentucky. On the basis of symptoms produced on cucumber he concluded that all samples except one were similar to Doolittle's virus (4). Porter referred to these isolates as "cucumber virus 1." The isolate which differed from the others was called "cucumber virus 2"; this was obtained from Bettendorf, Iowa. In addition to differences in symptoms on cucumber (var. White Spine) the latter virus infected watermelon, West India gherkin (*Cucumis anguria* L.), and African citron (*Citrullus vulgaris* Schrad). Recently Price (28) has shown by immunity studies the similarity of Porter's "cucumber virus 1" and Doolittle's cucumber virus 1.

Hoggan (10) has described a "yellow cucumber mosaic" virus which differed from cucumber virus 1 in producing a bright yellow mottle on tobacco and other hosts, but it had identical properties, modes of transmission, and host range. It was considered a variant of cucumber virus 1. A second virus, "cucumber mild mosaic," was described by her as producing milder symptoms and being lower than cucumber virus 1 in thermal inactivation point, tolerance to dilution, and longevity in vitro. She considered that this virus also belonged to the cucumber mosaic virus group.

Price (24) was able to isolate a number of yellow mosaic and necrotic type viruses from the bright yellow spots which appeared on the leaves of tobacco infected with Porter's (22) "cucumber virus 1." Each strain was found to produce different symptoms on several hosts, but all were similar in regard to thermal death point and aging in vitro. In another experiment in which cucumber mosaic virus received from E. M. Johnson was passed from primary lesions on cowpea in successive transfers, Price obtained evidence that the virus may occasionally become altered. This occurred when certain of these transfers on cowpea developed both yellow and necrotic lesions. Subsequent transfers to cowpea from the yellow lesions again produced yellow primary lesions, a severe systemic mottle, and also a yellow mosaic in tobacco.

In England Ainsworth (1) has described three mosaic diseases of cucumber. Two of these, which he refers to as "green mottle mosaic" or "cucumber virus 3" and "yellow mosaic" or "cucumber virus 4," respectively, occur naturally only on cucumber and differ in host

range from the third, "yellow mottle mosaic" ("cucumber virus 1"), since they induce systemic infection of watermelon, but they are not infectious to solanaceous plants. He reports that they also differ from cucumber virus 1 in ability to resist aging in vitro for 9 months or longer, and to survive heating for 10 minutes at 80° C., though they are inactivated at 90°. The "yellow mosaic" virus differs from "green mottle mosaic" only in symptoms produced on cucumber and is regarded as a strain of the latter. Ainsworth regards cucumber viruses 3 and 4 as distinct from cucumber virus 1, the last-named being identical with Johnson's (15) cucumber virus 1.

It has been shown that viruses of the cucumber mosaic group are the causal agents of a number of common diseases of plants other than cucurbits. Hoggan (9) gave evidence that the disease originally described as "spinach-blight" by McClintock and Smith (19) is probably identical with cucumber mosaic (cucumber virus 1) on spinach. Wellman (33) has described a virus causing a serious disease of celery (celery virus 1) with a much wider host range than cucumber virus 1, but it has physical properties and symptoms similar to those of the latter virus. More recently Price (26) in his immunity studies has given further proof of the close relation between celery virus 1 and his strain of cucumber mosaic virus. He has also shown (27) that the viruses of lily mosaic, ordinary cucumber mosaic, strain 6 of cucumber mosaic, and celery mosaic produce similar symptoms on lily, and that zinnia leaves infected with the passage strain of lily mosaic virus are immune to infection from strain 6 of cucumber-mosaic virus. Wellman (34) has also obtained infection on banana with celery virus 1, resulting in symptoms similar to bunchy top of banana. Köhler (18), working in Germany, described a lupine disease which he concluded was caused by Ainsworth's (1) yellow mottle mosaic virus of cucumber (cucumber virus 1). Harter (7) obtained a virus from lima bean which he concluded belonged to the cucumber mosaic virus group.

The cucumber mosaic viruses occurring in the United States, reported above, are shown to have certain definite similarities, and those which were studied most critically gave further evidence that they belong to the same group. From the present information it appears that there are a large number of closely related viruses which may differ greatly in symptoms and hosts affected. However, cucumber virus 3 and cucumber virus 4 reported from England (1) appear to be distinct from cucumber virus 1 and, therefore, probably do not belong to the same group.

Strain 14, described in this paper and found occurring naturally on pea and bean, has a host range that parallels, yet exceeds, that of cucumber virus 1. In property studies the two viruses are similar, and on certain plants symptom differences are hard to distinguish. The principal difference is the ability of strain 14 to infect systemically kidney bean, Fordhook lima bean, pea, jimsonweed, cowpea, and yellow sweetclover. Cucumber virus 1 and celery virus 1 cannot be considered infectious on pea, but the fact that symptoms developed on a few plants inoculated with these viruses suggests the possibility of obtaining strains from these viruses that readily infect pea. The work of Price (24, 27), who obtained a strain of cucumber mosaic systemic on cowpea and a passage strain of the lily-mosaic virus on tobacco, lends support to this hypothesis, and furnishes a plausible

explanation for the origin of strains 14 and 17. Strain 14 resembled more nearly celery virus 1 in symptoms, property studies, and host range than did any of the other viruses studied. However, there were certain hosts that were infected by strain 14 but not by celery virus 1, and vice versa.

Strain 17, also described in this paper, produces symptoms on tobacco and cucumber which are nearly identical with those of "cucumber mild mosaic virus" described by Hoggan (10). The properties of the two viruses do not differ significantly. Strain 17 has a host range which differs from that of cucumber virus 1 only in the ability of the former to infect pea, jimsonweed, and sweetclover systemically. Only in symptoms do the two viruses differ appreciably.

At present the natural occurrence of strains 17 and 14 has been limited to pea and bean plantings near Madison, Wis. Diseased pea plants from various parts of Wisconsin showing similar symptoms were not infected with these viruses. Whether the limited occurrence of these strains on legumes is due to their recent origin or to environmental factors is at present only a matter for speculation. Harter's (7) report of a cucumber mosaic virus on lima bean in Maryland and Zaumeyer's (39) discovery of two viruses of this group on pea in Colorado suggest a widespread occurrence of cucumber mosaic viruses on legumes. Sweetclover, which is a host of strains 14 and 17, supplies a common biennial legume in which the viruses may overwinter.

Strains 14 and 17 differ distinctly from the viruses of bean mosaic described by Pierce (21) and the pea viruses reported by Stubbs (30) in host range, properties, and symptoms on certain hosts. Zaumeyer and Wade (40, 41) and more recently Zaumeyer (38) have reported a number of viruses affecting pea and bean. These also differ in either host range, properties, or symptoms. However, two of these (alfalfa viruses 1A and 1B) (38) are of special interest since they infect systemically a number of hosts susceptible to strains 14 and 17, including tobacco, zinnia, and cucumber. The properties of alfalfa-mosaic viruses 1A and 1B, including thermal inactivation point, longevity, and tolerance to dilution, are within the range reported by several workers for strains of cucumber-mosaic virus, but because of the differences in symptoms and host range it is not probable that they are related to cucumber virus 1. Nevertheless immunity studies similar to those described by Price (25) and other direct comparisons of the viruses might prove of value in demonstrating more distinct differences or similarities.

On the basis of the present information viruses 14 and 17 appear to be as closely related to cucumber virus 1 as a number of other viruses previously reported. Whether they should be regarded as strains or as related viruses is largely a matter of definition. In this paper they have been referred to as strains primarily for the convenience of placing them in the cucumber mosaic virus group, of which cucumber virus 1 is referred to as the type virus.

#### SUMMARY

The two viruses discussed in this paper, one affecting peas and the other both peas and beans, were found inducing disease on these plants in nature. Since this investigation has given evidence that the two viruses are related to cucumber-mosaic viruses they are tentatively referred to as strains 14 and 17 of that group.



The symptoms produced on peas and beans are described in some detail. Strain 14 infected all 25 strains of bean and the 3 varieties of pea tested. Minor varietal and strain differences were observed and examples of these are given. Strain 17 infected the 3 varieties of peas tested but none of the 25 strains of bean. Neither cucumber virus 1 nor celery virus 1 infected any of the above varieties of bean systemically, but a few pea plants inoculated with these viruses developed local symptoms. However, infection could not be obtained in successive experiments, and for this reason peas are not regarded as hosts of the last-named viruses.

Three substrains of 14 were isolated; two of these remained stable and produced distinct symptoms on tobacco, cucumber, bean, pea, and cowpea. The "yellow" isolate produced a more severe disease while the "dark green" isolate was less virulent than the original strain.

On plants infected with strain 14 the disease was most severe at temperatures of 24° to 28° C., within which range stunting, mottling, and necrosis occurred. Inoculated plants held at 16° developed no symptoms except stunting and epinasty of inoculated primary bean leaves.

The properties of strains 14 and 17 were found to agree in general with those described for several cucumber-mosaic viruses. Strains 14 and 17 remained infectious *in vitro* 7 days at 20° to 22° C. The tolerance to dilution is about 1 to 10,000 for strain 14 and 1 to 1,000 for strain 17. The thermal inactivation point for strain 14 is about 65° C. and for strain 17 between 65° and 70°.

The two viruses were readily transmitted by means of the peach aphid to and from a number of host plants. Mechanical transmission was also accomplished with or without the use of carborundum powder as an abrasive. No seed transmission was obtained in 580 pea and 440 bean seedlings grown from seed of plants infected with strain 14. Seed transmission was not studied with strain 17.

Table 3 gives a summary of the hosts infected and cardinal symptoms produced by the 4 viruses. This list includes 19 species belonging to 16 genera and 6 families. Strains 14 and 17 infected all plants susceptible to cucumber virus 1, and strain 14 was pathogenic on a number of additional hosts. Strain 17 differed from cucumber virus 1 only in systemic infection on pea, jimsonweed, and yellow sweet-clover. The host range of strain 14 closely paralleled that of celery virus 1 on the plants tested, but each virus infected certain hosts not susceptible to the other. On some hosts the symptoms of strains 14 and 17 were similar to those of cucumber virus 1, but on other hosts certain differences were evident. Soybean (var. Manchu) and White Dutch clover were not infected by any of the 4 viruses.

Preliminary immunity studies give further indication of the close relationship of strain 14 to the cucumber mosaic group.

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# TULIP ANTHRACNOSE <sup>1</sup>

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## INTRODUCTION

An anthracnose disease, affecting primarily the peduncles and leaf blades of Darwin tulips (*Tulipa gesneriana* L.) was found in a garden at Burlingame, Calif., in April 1939. A preliminary report <sup>2</sup> of the disease has already been given. The symptoms of the disease and the identity of the causal organism are discussed in this paper.

## SYMPTOMS OF THE DISEASE

Symptoms of the disease on Rev. H. Eubank and Zwanzenburg, the only varieties of the tulip known to be susceptible to natural infection, were identical in all respects. They consisted of small to large elliptical lesions, 0.5 to 2 cm. in length and 0.2 to 1 cm. in width, on both peduncles (fig. 1) and leaf blades. In general, the long axes of the lesions were parallel to the long axes of peduncles and leaf blades. In the early stages of infection, the lesions are water-soaked; later, they become dry and black around the margins. Numerous small, black acervuli develop in the central part of the lesions.



FIGURE 1.—Natural infection of tulip peduncles, showing elliptical lesions containing small, black acervuli. Collected at Burlingame, Calif.

## THE PATHOGEN

The causal fungus was readily isolated on potato-dextrose agar either by tissue plantings from peduncles or leaf blades, or by direct

<sup>1</sup> Received for publication August 14, 1940. Contribution from the Division of Plant Pathology, California Agricultural Experiment Station.

<sup>2</sup> TOMPKINS, C. M., and HANSEN, H. N. TULIP ANTHRACNOSE. (Abstract) *Phytopathology* 30: 790. 1940.

transfer of spores from the lesions. In either case, the fungus sporulates very sparsely. Only a few tiny acervuli are produced on the surface of the medium, and they are entirely covered by a profuse growth of aerial mycelium. Like many other imperfect fungi, this fungus appears to occur in nature in the dual condition,<sup>3</sup> for, when 50 single-spore cultures were made, 3 culture types appeared, 1 producing acervuli and conidia in great abundance with little or no aerial mycelium (fig. 2, *B*), 1 producing few conidia and abundant mycelium (fig. 2, *C*), and 1 like the parent (fig. 2, *A*).

Young tulip plants, varieties Rev. H. Eubank and Zwaneburg, were placed in a moist chamber for 24 hours and then inoculated by



FIGURE 2.—Three culture types of the pathogen: *A*, The primary isolate; *B*, the conidial type; *C*, the mycelial type derived by single-sporing from *A*.

atomizing with a heavy suspension of spores in water. The inoculated plants were then returned to the moist chamber for an additional 24 hours, after which they were placed on a bench in the greenhouse. Control plants were not atomized. Infection, as indicated by typical lesions on peduncles and leaf blades (fig. 3, *A*, *B*), was obtained in 7 to 10 days. Under greenhouse conditions, the flowers were also infected. Additional, successful inoculations were made by atomizing the plants directly on benches in the greenhouse, without using a moist chamber. In all cases, the symptoms produced by artificial inoculation were identical with those produced in nature. The control plants remained healthy. The fungus was reisolated, and the reisolates proved pathogenic by inoculation. No infection was obtained by inoculation of the variety Clara Butt which, together with Fantasy, appears to be immune.

It was observed that the first two culture types (fig. 2, *B*, *C*) upon single-sporing remained constant, whereas the parent type (fig. 2, *A*)

<sup>3</sup> HANSEN, H. N. THE DUAL PHENOMENON IN IMPERFECT FUNGI. *Mycologia* 30: 442-455, illus. 1938.

again gave rise to the three types. On tulip, the fungus is a typical *Gloeosporium*, producing conidia in well-defined acervuli from 0.2 to 1.5 mm. in diameter. The conidia vary greatly in size from  $9.75\mu$  to  $23.75\mu$  by  $3.25\mu$  to  $7.25\mu$ , with the greater number measuring about  $15.0\mu$  by  $5.0\mu$ .

In reading descriptions of the many species of *Gloeosporium* in Saccardo,<sup>4</sup> one cannot fail to notice how closely many of the species

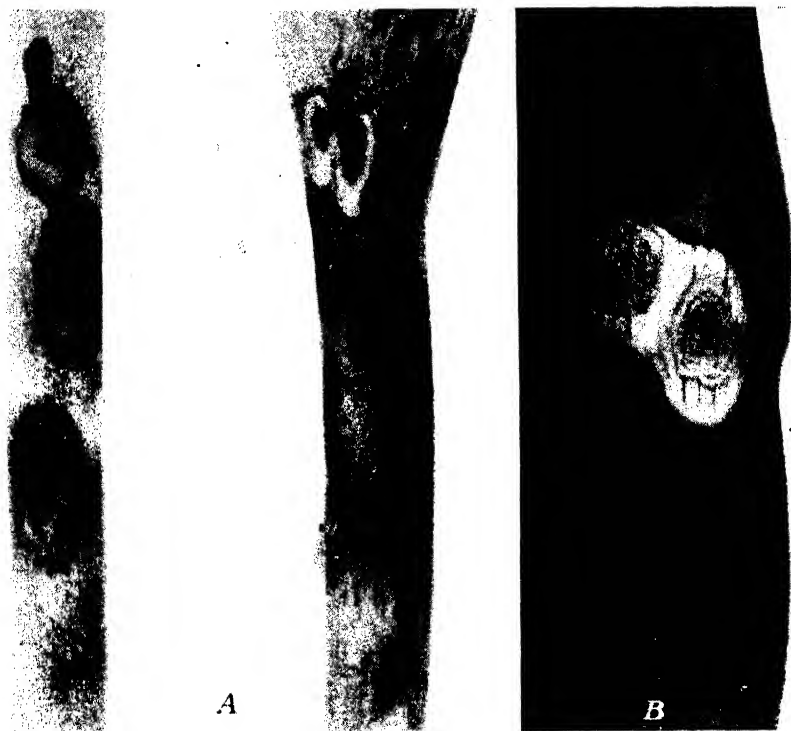


FIGURE 3.—Artificial infection of Zwanenburg tulips produced by atomizing with a water suspension of spores in the greenhouse: A, Infected peduncles; B, infected leaf blade. All lesions show a water-soaked margin, while the central areas contain numerous black acervuli.

resemble each other morphologically. Seemingly, habitat or host has in most instances served as the main basis for separation. Recognizing the inadequacy of such a system, it seems desirable to refrain from further encumbering the literature by adding a new species to a genus already so numerously represented. The lead recently set in the taxonomic treatment of fusaria<sup>5</sup> will be followed. Thus, this fungus is considered to be a form of the oldest species of *Gloeosporium* occurring on a monocot, the description of which will fit within the limits of measurements given above. The name *Gloeosporium thumenii* Sacc. f. *tulipae* forma nov. is proposed.

The method of overwintering of the fungus has not been determined.

<sup>4</sup> SACCARDO, P. A. SYLLOGE FUNGORUM OMNIUM HUCUSQUE COGNITORUM. v. 3, p. 721. 1884.

<sup>5</sup> SNYDER, W. C., and HANSEN, H. N. THE SPECIES CONCEPT IN FUSARIUM. Amer. Jour. Bot. 27: 64-67. 1940.

## SUMMARY

Tulip anthracnose occurs at Burlingame, Calif.

Symptoms consist of elliptical lesions which at first are water-soaked, but later become dry and covered with fruiting bodies.

Infection of the varieties Rev. H. Eubank and Zwanenburg was obtained in 7 to 10 days by atomizing the plants with a water suspension of spores. The varieties Clara Butt and Fantasy appear to be immune in the field and in the greenhouse.

The name *Gloeosporium thumenii* Sacc. f. *tulipae* forma nov. is proposed.

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## THE INFLUENCE OF TEMPERATURE AND SEASON ON THE CITRUS RED MITE (*PARATETRANYCHUS CITRI*)<sup>1</sup>

By I. L. ENGLISH, *entomologist, Department of Zoology-Entomology, Alabama Agricultural Experiment Station*, and G. F. TURNIPSEED, *entomologist, Alabama Department of Agriculture and Industries*<sup>2</sup>

### INTRODUCTION

Several investigators have emphasized the importance of temperature in relation to the development and abundance of the citrus red mite (*Paratetranychus citri* McG.). Woodworth<sup>3</sup> observed as early as 1902 that there was a relationship between the population of this pest and the season of the year. Quayle<sup>4</sup> noted that the population of mites reached its greatest density during the spring months and largely disappeared in the summer. Boyce,<sup>5</sup> some years later, pointed out that infestations of this mite were distinctly limited by climatic factors and that it thrived best under cool, semihumid conditions. Quayle<sup>6</sup> presented a map showing that the regions of California in which the citrus mite is a serious pest are along the Pacific coast. At Los Angeles, for example, the mean monthly temperature is about 70° F. in July and August. At Riverside, where the mean monthly temperature for these 2 months is 75°, citrus mite occurs but is not an important pest. Further inland, at Lindsay, the mean monthly temperature is near 80° during July and August, and in this region the citrus mite is not known as a pest. Although the influence of temperature on the citrus red mite has been recognized, apparently no definite relationship between temperature and mite development has been established.

The experiments whose results are herein reported were carried out over a period of several years. Temperature observations, the time required for mites to develop from egg to adult, and the length of life of individual mites were recorded from 1930 to 1936, inclusive. After preliminary studies showed that a relationship existed between temperature and incubation and development, additional cultures were started in 1938 to obtain more complete data on life history, as well as further information on the variation of development with season. From these data an attempt was made to show that, although development is accelerated by increases in temperature, other factors promote population density in cool weather.

<sup>1</sup> Received for publication Dec. 11, 1939.

<sup>2</sup> Field laboratory, Spring Hill, Ala.

<sup>3</sup> WOODWORTH, C. W. THE RED SPIDER OF CITRUS TREES. Calif. Agr. Expt. Sta. Bul. 145, 19 pp., illus. 1902.

<sup>4</sup> QUAYLE, H. J. CITRUS FRUIT INSECTS. Calif. Agr. Expt. Sta. Bul. 214: [443]-512, illus. 1911.

<sup>5</sup> BOYCE, A. M. THE CITRUS RED MITE *PARATETRANYCHUS CITRI* M'G. IN CALIFORNIA, AND ITS CONTROL. Jour. Econ. Ent. 29: 125-130. 1936.

<sup>6</sup> QUAYLE, HENRY J. INSECTS OF CITRUS AND OTHER SUBTROPICAL FRUITS. 583 pp., illus. Ithaca, N. Y. 1938.



## METHODS

The rearing cells constructed of felt and celluloid described by Newcomer and Yothers<sup>7</sup> were used for observing individual mites. From 1930 to 1936, inclusive, cells half an inch in diameter were used. During 1938 the size of the cells was increased to 1 inch to provide more freedom and food for the individuals. The cells were fastened to the leaves of small Satsuma trees grown in 12-inch pots in a roofless, screened insectary. The portion of the leaf enclosed by the cell was drawn in a notebook, and daily records of oviposition, hatching, etc., were kept.

Weather records were obtained from maximum and minimum thermometers and a hygrothermograph in an instrument shelter nearby. Cutright,<sup>8</sup> and others as well, have recognized the limitations in the use of the mean of the daily maximum and minimum temperatures for studying biological phenomena. His method of calculating

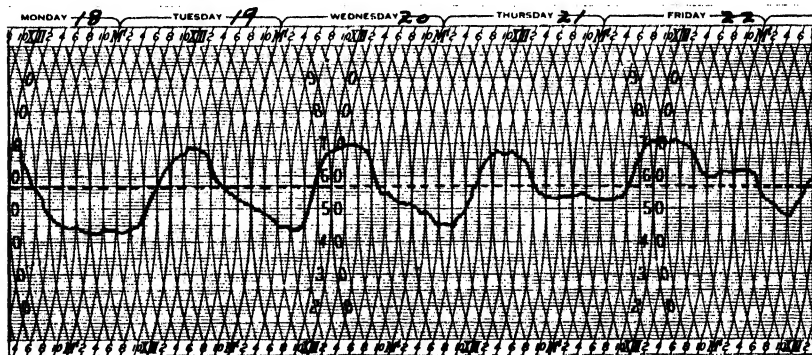


FIGURE 1.—Thermograph chart with calculated average temperature indicated by dotted line.

mean temperatures from planimeter measurements of thermograph charts was simplified somewhat by the use of the following formula:

$$T = \frac{RA}{A'} + t, \text{ where}$$

$T$  = the average temperature,

$R$  = the temperature range of the thermograph chart,

$A$  = the area in square centimeters under the temperature curve for the period of observation (measured with a planimeter),

$A'$  = the area of the chart in square centimeters for the period of observation (calculated from the dimensions of the chart),

$t$  = the minimum recordable temperature on the chart.

The average temperature obtained from a mean of the temperature values at 2-hour intervals on the chart in figure 1 was 56.61. The average temperature computed by the above formula was 56.48. Theoretically, the formula gives the temperature value which would be obtained by taking the mean of an infinite number of observations.

In actual practice the temperature area was obtained from identical charts, and every period of development was recorded as "average

<sup>7</sup> NEWCOMER, E. J., and YOTHERS, M. A. BIOLOGY OF THE EUROPEAN RED MITE IN THE PACIFIC NORTHWEST. U. S. Dept. Agr. Tech. Bul. 89, 70 pp, illus. 1929.

<sup>8</sup> CUTRIGHT, C. R. NOTES ON THE COMPUTING OF MEAN TEMPERATURES FOR BIOLOGICAL USE. Ent. Soc. Amer. Ann. 20: 255-261, illus. 1927.

daily area." The conversion of area to average temperature is then reduced to

$$T = \frac{90 \times A}{7.6 \times 4.0} + 10 \\ = 2.96A + 10.$$

The temperature values which were plotted on cross-section paper were the average temperatures corresponding to individuals with identical developmental periods. For example, records were obtained on 12 individuals which developed from egg to adult in 15 days. Therefore, the average temperature for the 12 developmental periods was plotted against 15 days. The constants of equations and the curves representing the relation between average temperature and the developmental period were calculated by the method of least squares.

## RESULTS

### THE EFFECT OF TEMPERATURE AND SEASON ON THE INCUBATION PERIOD

The incubation periods of 1,895 eggs were observed over a period of 8 years. When the incubation period was plotted against the average temperature on semilogarithmic paper, the points fell approximately along a straight line, indicating that the exponential equation  $y = ae^{-bx}$  would express the relationship (fig. 2). In this equation  $y$  is the incubation period in days,  $x$  is the average temperature in degrees Fahrenheit, and  $a$  and  $b$  are constants. The value found for  $a$  was 1,125.8, and for  $b$ , 0.073. Hence the relationship between the incubation period and average temperature is expressed by the equation  $y = 1,125.8e^{-0.073x}$ .

This equation is used for estimating the incubation period. Suppose the area under a temperature curve for an estimated incubation period of a week was measured and the average temperature was found to be 64.3° F. Substituting 64.3 for  $x$ , the equation becomes  $y = 1,125.8e^{-4.69}$ . From an exponential table it is found that  $e^{-4.69} = 0.0092$ . Then  $y = 1,125.8 \times 0.0092$ , or 10.3. The observed value was 10 days.

When the average incubation period was plotted against the time in months from January to December, a U-shaped curve resulted (fig. 3). The incubation period averaged 20.4 days for eggs deposited in January. During May, June, July, August, and September the average ranged from 5 to 6 days. Eggs deposited in December required an average of 23.4 days to hatch, indicating that they were exposed to more cold weather subsequent to deposition than those deposited in any other month.

### THE EFFECT OF TEMPERATURE AND SEASON ON DEVELOPMENT FROM EGG TO ADULT

A study of the data from 56 males and 176 females which were observed from egg to adult revealed that the relationship between the time of development and average temperature was also exponential. Data on males and females were examined separately but no significant difference in their rates of development was found. The equation and curve were developed from the combined data on males and females (fig. 4). The equation which expresses the relationship be-

tween average temperature and the time required for development from egg to adult is  $y = 739.35e^{-0.0564x}$ , where  $x$  is the average temperature and  $y$  the period of development.

Only 10 or 12 days are required for development from egg to adult in the summer. The periods are much longer in the winter months

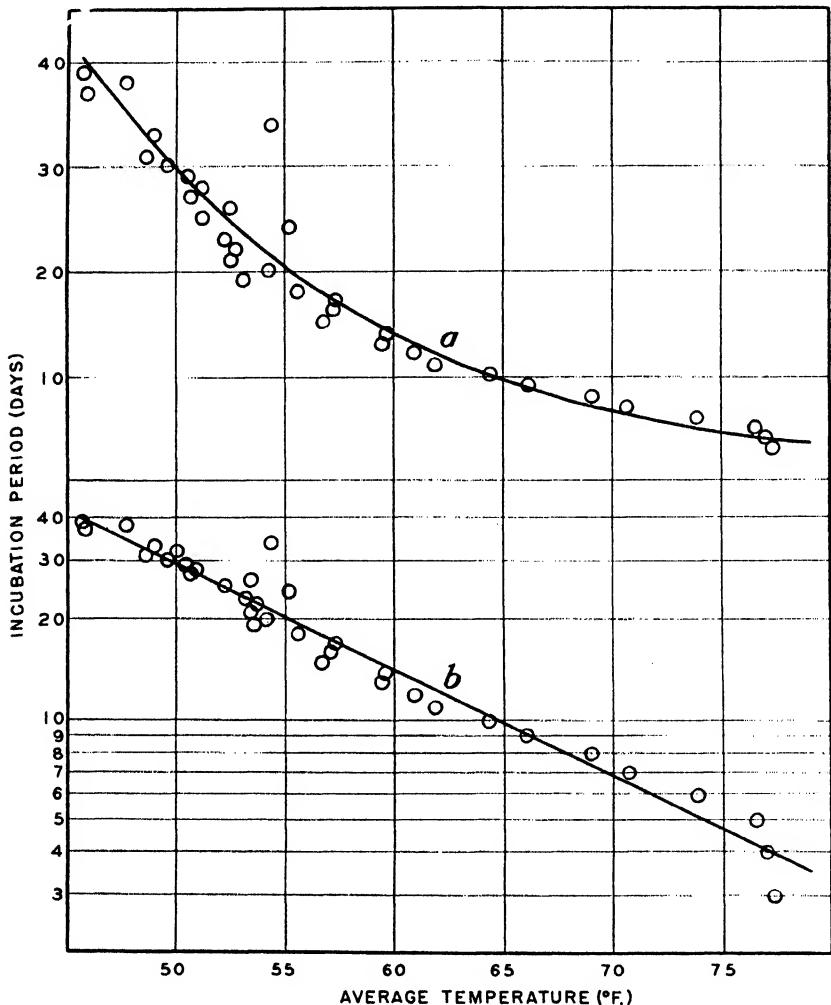


FIGURE 2.—The relationship between average temperature and the length of the incubation period: *a*, Data plotted on cross-section paper; *b*, data plotted on semilogarithmic paper.

and an extreme of 50 days was recorded for 1 female. The average length of these developmental periods forms a U-shaped curve as the season proceeds from January to December (fig. 5). An average of 34.5 days was required for egg to adult development from eggs deposited in January. In February this average dropped to 26.2, and

by March to 19.9 days. Broods beginning in May, June, July, and August required 12 days or less for development, but by November the requirement had advanced to more than 30 days.

#### DEVELOPMENT FROM EGG TO EGG

After becoming adult, females may spend from 3 to 20 days, or even more, before depositing the first egg. The time spent in the period of preoviposition varies greatly with the season, just as other periods of development vary. The life cycle ranged from 13 to 15 days in the summer to 61 days for a brood beginning in December.

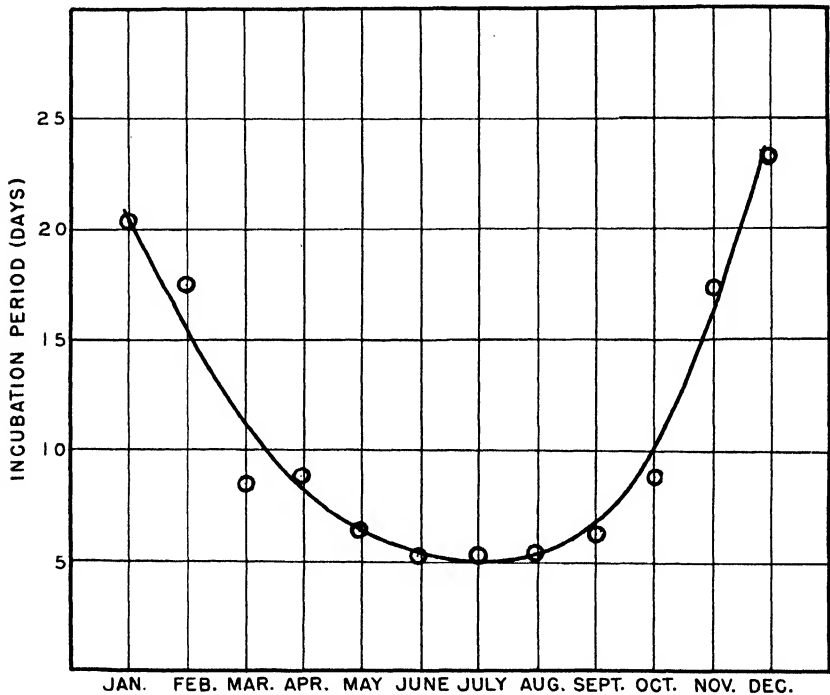


FIGURE 3.—The relationship between the season and the average length of the incubation period.

A study of the length of the life cycle of females and the average temperature disclosed that the data approximated a straight line when plotted on semilogarithmic paper (fig. 6). The equation derived from these data was  $y = 1,305.6e^{-0.0007x}$ , where  $y$  is the time in days from egg to egg and  $x$  is the average temperature.

#### THE ADULT LIFE SPAN

The outstanding difference in the life history of male and female mites is the length of life after reaching the adult stage. Adult males originating in June, July, and August, lived only an average of 5 or 6 days, while females lived about 13 days in the adult stage. The average adult life span of 126 females was 21.8 days and that of 55 males was 14.5 days. The seasonal differences are shown in figure 7.

The influence of temperature on the adult life span was not so marked as on development, but it was evident that a general correlation existed. A straight line, rather than an exponential curve, appears to be a valid expression of the relationship between average

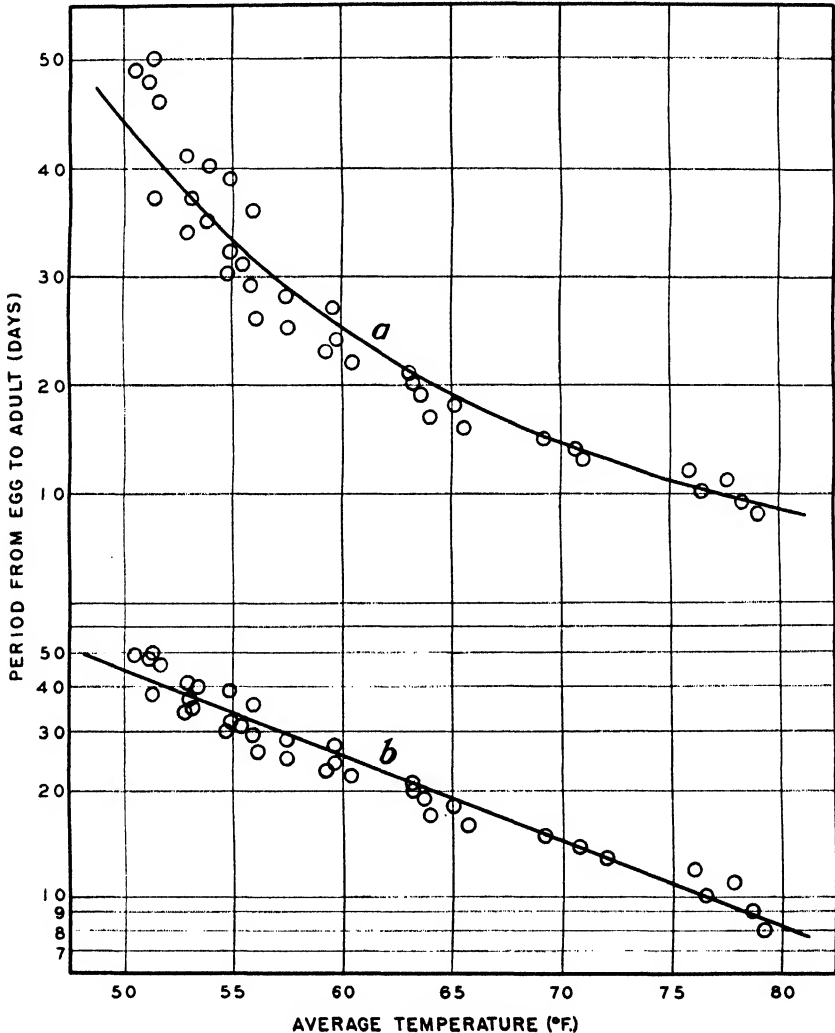


FIGURE 4.—The relationship between average temperature and development for the period from egg to adult: *a*, data plotted on cross-section paper; *b*, data plotted on semilogarithmic paper.

temperature and the adult life span (fig. 8). The line fitted to the data on males has the equation  $y = -1.13x + 94.44$ , and that for females has the equation  $y = -1.40x + 117.25$ , where  $y$  is the adult life span in days and  $x$  is the average temperature.

## THE TOTAL LIFE SPAN

Studies were made of the average temperatures of birth-to-death periods of both males and females but no satisfactory relationship was established. Since the time of development from egg to adult

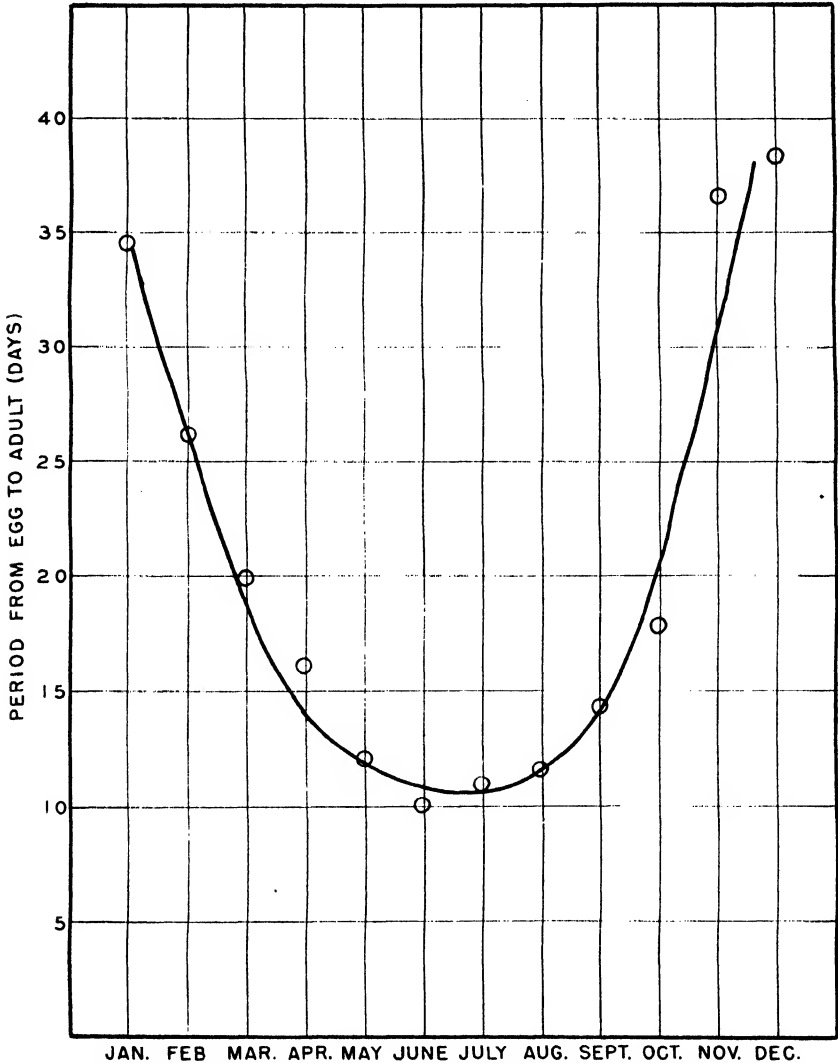


FIGURE 5.—The relationship between season and development for the period from egg to adult.

was found to be an exponential function of average temperature, while the adult life span was a linear function, it is evident that, although there is a relationship between the birth-to-death period and average temperature, it cannot be expressed by a simple equation.

The difference in the total life span of the sexes arises mainly from the difference in the longevity of the adults. The average total life span of females varied from 39.3 days for those originating in January to 16.6 in July and 60.3 in November. Males averaged 34.0 days for

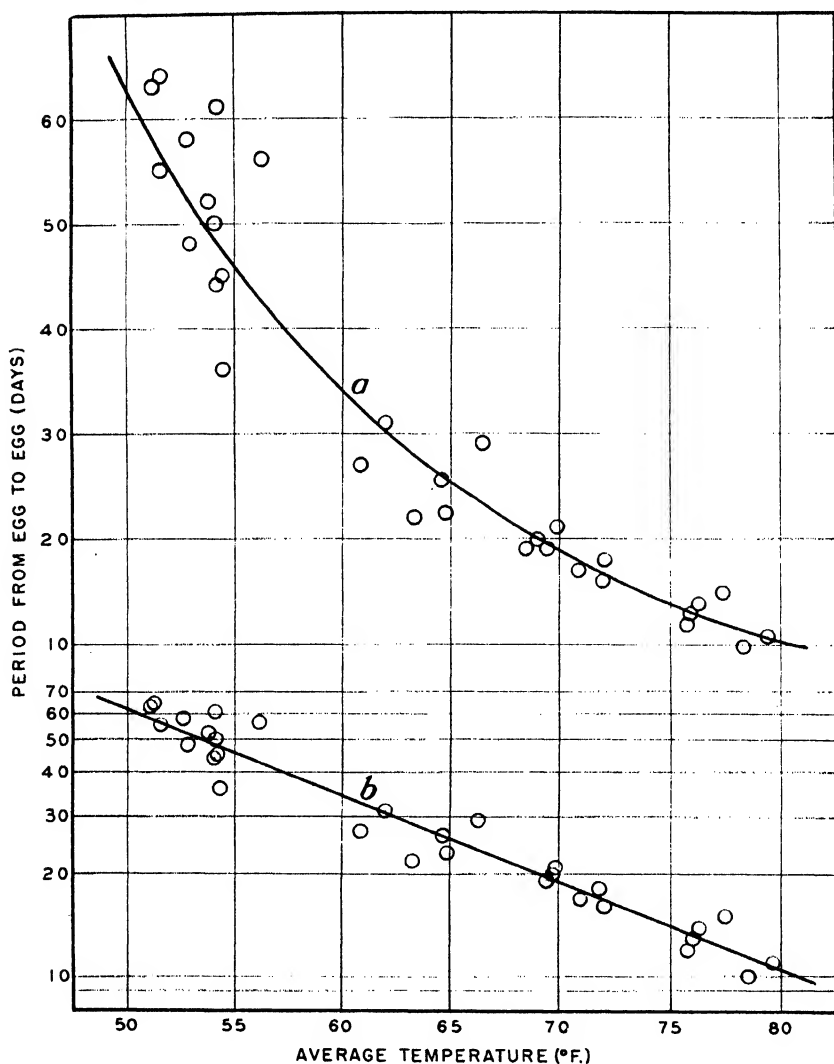


FIGURE 6.—The relationship between average temperature and development for the period from egg to egg: *a*, Data plotted on cross section paper; *b*, data plotted on semilogarithmic paper.

January and declined to a minimum of 10.2 days for June, but one male which originated in November lived for 94 days (fig. 9). The average length of life for 125 females over all seasons was 30.4 days; the average for 55 males was 22.9 days.

## THE RELATIONSHIP BETWEEN OVIPOSITION AND THE ADULT LIFE SPAN OF FEMALES

The curve representing the adult life span of females originating in the months of January to July, inclusive, was very similar to the curve representing the number of eggs deposited (fig. 10). The average adult life declined from 38.0 to 13.2 days for this period, and the average number of eggs deposited ranged from 39.0 to 15.8 per female. It will be observed that the average number of eggs laid exceeded the average number of days spent in adult life. Hence, more than 1 egg per day was deposited. The maximum rate was 1.50

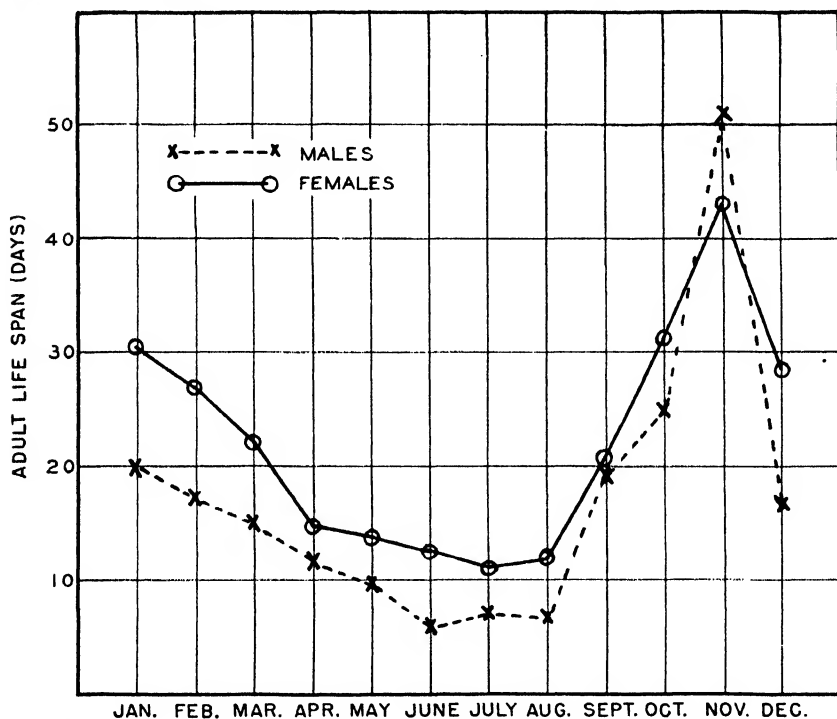


FIGURE 7.—A comparison of the adult life span of males and females in different seasons.

per day by females originating in February. Beginning with August, the egg-curve dropped below the life-span curve and remained below it the rest of the calendar year. Oviposition reached the minimum rate of 0.21 eggs per day by females originating in October. The data used are for the year 1938 and the value plotted for December is probably in error, since only one individual originated in this month.

## THE VARIATION OF MITE POPULATION WITH THE SEASON

From monthly egg counts on 10-cm.<sup>2</sup> disks punched from untreated leaves an indication of the variation of mite population with the season was obtained. A curve was prepared from the average of



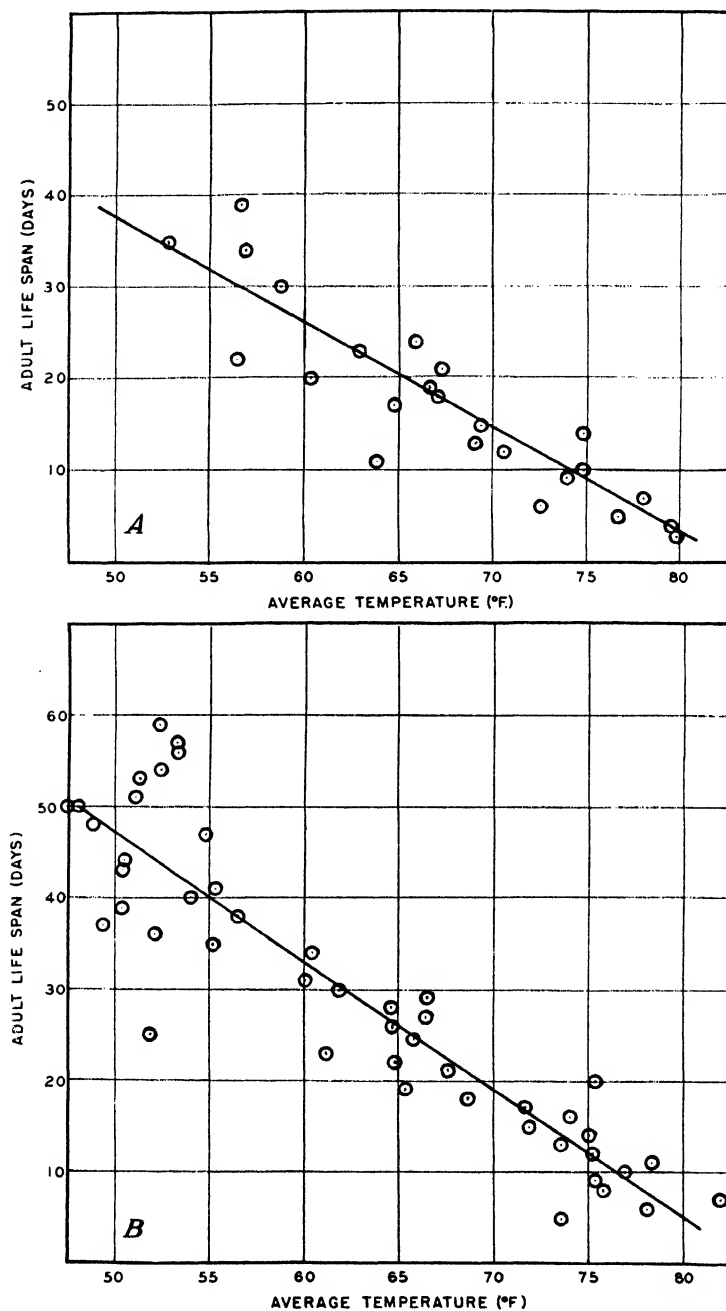


FIGURE 8.—The relationship between average temperature and the adult life span of males (A) and females (B).

counts made during 6 years (fig. 11). Ordinarily the population of mites is highest during March, April, and May. It will be observed that there was a gradual decline in the number of eggs as warm weather approached. The peak indicated at the last of June was obtained from an unusually heavy infestation which developed 1 year out of the 6. It is believed that this was exceptional. During the hot months of July, August, and September, egg counts were very

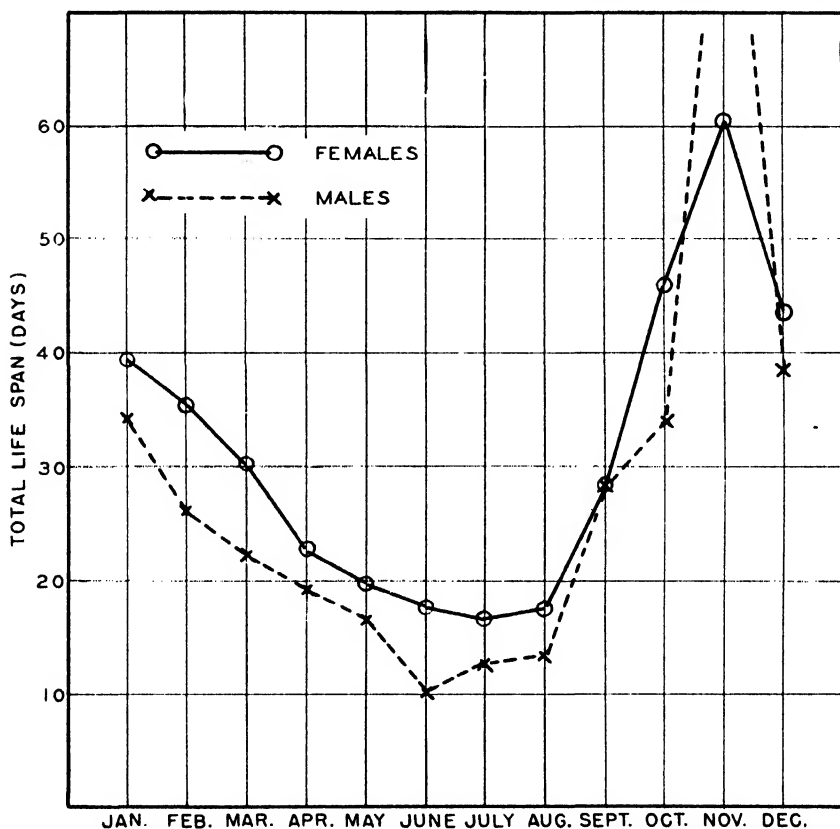


FIGURE 9.—The relationship between the season and the total life span (birth to death) of males and females.

low, indicating unfavorable conditions for mites. With the advent of cool weather in the fall the population began to build up again.

Inasmuch as the development of the citrus red mite has been shown to be a function of temperature, it may be asked why the population does not reach its greatest density in the summer. Although the time required for the incubation of eggs and development is shortest in the summer, other factors are of more importance. For example, at 50° F. the calculated time for development from egg to adult is 44.1 days; at 80° the time is 8.1 days. Hence, development at 80° is 5.4 times as fast as at 50°. But the adult life span is more than 9 times as long at 50° as at 80° (fig. 8). Thus the rate of development in hot

weather is more than offset by the longer life in cool weather. This factor alone would account for an increase in the population in cool weather. To increase further the population potential for cool weather is the fact that during the season of greatest population density more eggs per female are deposited (fig. 10). In addition, high temperatures are probably lethal to the mites, since if the curves in figure 8 were extrapolated the thermal death-point would fall at about an average temperature of  $85^{\circ}$ .

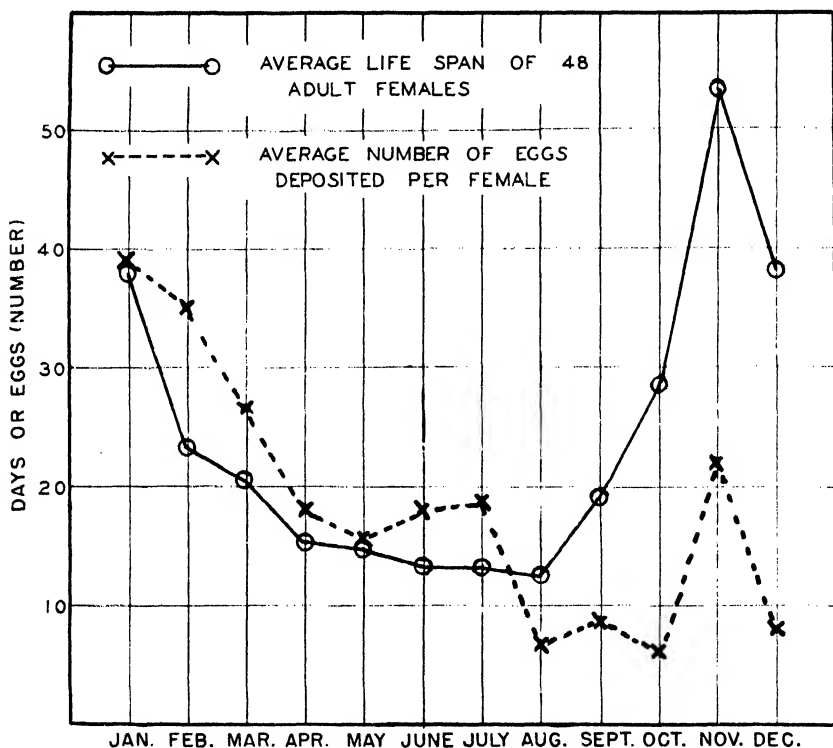


FIGURE 10.—The relationship between the season, the average life span of adult females, and the average number of eggs deposited.

In spite of favorable temperature conditions in the fall, mite populations do not usually become dense at this season of the year. In the first place, the population must recover the momentum lost in the summer. In the second place, the rate of egg production per female is low. It is possible, too, that the quality of the food is an important influence on mite population. In the fall of the year trees have a load of ripening fruit and at the same time are entering a semidormant stage. It will be noted that the population becomes dense in the spring when fertilizing and cultural practices usually have been started in the orchards and the trees assume vigor. These factors, in addition to temperature conditions, are probably favorable to the mite.

## SUMMARY

Several phases of the life history of the citrus red mite were found to be mathematically related to the average temperature computed from thermograph charts. The length of the incubation period, the time for development from egg to adult, and the time for development from egg to egg were found to be functions of average temperature, which could be approximated by the exponential equation  $y = ae^{-bx}$ , where  $y$  is the period of time in days and  $x$  the average temperature in degrees Fahrenheit. The duration of adult life was found to be a linear function of average temperature, conforming to the equation  $y = -ax + b$ , where  $y$  is the time in days and  $x$  is the average temperature.

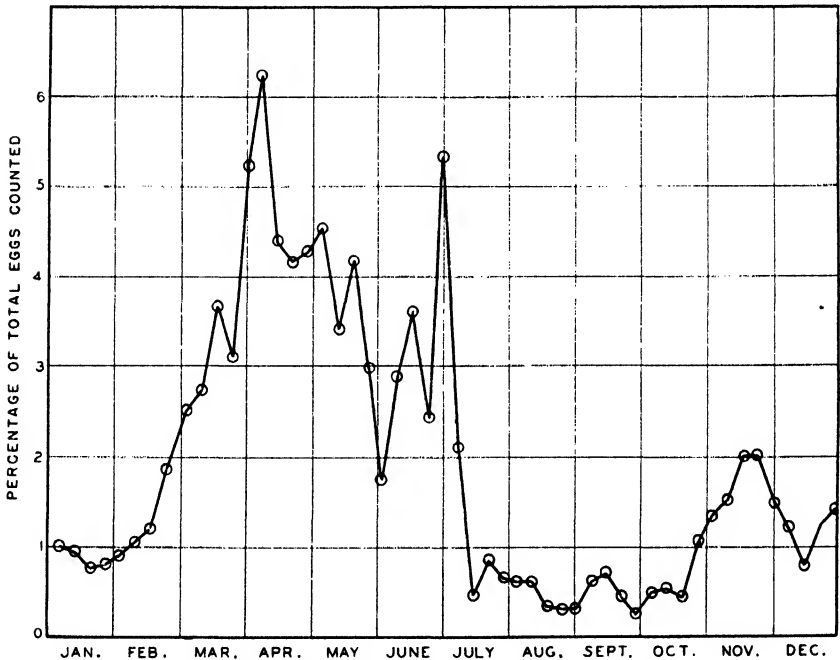


FIGURE 11.—The seasonal density of citrus red mite as indicated by egg counts.

The average incubation period and the time for development from egg to adult formed U-shaped curves when plotted against the months from January to December. The adult life span and the total life span of mites also approximated U-shaped curves when plotted against the months of the year. The adult life span of males was found to be shorter than that of females.

The population of the citrus red mite declines in hot weather and increases in cool weather. The early spring months apparently afford optimum conditions. Although the temperatures at this season do not induce the most rapid development, other factors, such as the long life of the adults and the high rate of egg production, aid in promoting the maximum population.



# CYCLOCEPHALA (OCHROSIDIA) BOREALIS IN CONNECTICUT<sup>1</sup>

By J. PETER JOHNSON

Assistant entomologist, Connecticut Agricultural Experiment Station

## INTRODUCTION

Since grubs of annual species of scarabaeidae have become more prevalent and injurious to turf in the northeastern part of the United States during the last two decades, it is increasingly important that the biology and description of such pests be made available to economic entomologists.

*Cyclocephala borealis* Arrow<sup>2</sup> has recently become a serious pest of lawns in Connecticut. In order to determine how to combat it successfully, the studies described below have been carried on over the past 3 years.

*Cyclocephala borealis* occurs as far south as Alabama and westward to California,<sup>3</sup> its range extending over most of the United States. It was found in Connecticut for the first time when grubs collected from injured turf on an estate in Westport (4),<sup>4</sup> November 12, 1931, were identified as larvae of this scarabaeid.

During October 1936, two different lots of grubs were received for identification from Greenwich, where severe lawn damage had occurred. In the fall of 1937, grubs were received from East Norwalk, Fairfield, and Greenwich. Upon investigation it was found that approximately 15 acres of lawn area had been badly injured or destroyed by the insect. In 1938, additional infestations were found in Greenwich and Norwalk, and another was reported from East Hartford. Other infestations were found in 1939 on two golf courses in Fairfield and on one course in Stamford. It is evident from the information at hand that a scattered infestation exists in the shore towns in southwestern Connecticut.

This insect is usually a pest of grassland, and a brief account of its injuriousness to lawns in Ohio, together with certain experiments in control methods, has been given by Neiswander (8). It has also been reported by Swenk (11) as injurious to winter wheat in Nebraska.

Certain morphological and biological studies of *Cyclocephala* (*Ochrosidia*) *borealis* have been made in recent years. Saylor (9) has described the adult male and published illustrations of the male genitalia. Sim (10) has described the larva and illustrated the epipharynx and raster. Neiswander (8) also reported on the life history of this insect. The biology of *C. (villosa) borealis* as reported by Hayes (6) in 1918 was really that of *C. immaculata* Oliv. (7, p. 67).

<sup>1</sup> Received for publication June 15, 1940.

<sup>2</sup> Dr. E. A. Chapin, U. S. National Museum, confirmed the identification of this scarabaeid as *C. borealis* Arrow from duplicate material submitted to him.

<sup>3</sup> U. S. DEPARTMENT OF AGRICULTURE, BUREAU OF ENTOMOLOGY AND PLANT QUARANTINE. THE INSECT PEST SURVEY BULLETIN. 1930, 1931, 1934, 1937, 1938. [Mimeographed.]

<sup>4</sup> Italic numbers in parentheses refer to Literature Cited, p. 86.

## SYSTEMATIC POSITION

This scarabaeid was originally described by Burmeister, under the subfamily Dynastinae, tribe Cyclocephalini, as *Cyclocephala villosa*. Since two species received the name of *Cyclocephala villosa*, the first being Blanchard's Bolivian species and having priority, Arrow (1, p. 172) renamed the North American species *borealis*. Casey (5, pp. 109-147), in his review of the subfamily Dynastinae, placed *villosa* in the genus *Ochrosidia*. Arrow considers *Ochrosidia* as a subgenus. The Leng Catalogue (2, p. 54) lists the species as *Cyclocephala borealis* Arrow.

## DESCRIPTION AND LIFE HISTORY

## THE EGG

The eggs of *Cyclocephala borealis* when laid are pearly white, ovoid, and delicately reticulate. The sides of some are nearly parallel, with rounded ends. When first laid, 15 eggs had a mean length of  $1.68 \pm 0.02$  mm. and a mean breadth of  $1.3 \pm 0.01$  mm. The eggs begin to swell when 2 or 3 days old, reaching a maximum size just before hatching. Eight other eggs measured 1 day before hatching had a mean length of  $1.68 \pm 0.03$  mm. and a mean breadth of  $1.58 \pm 0.04$  mm. It will be noted that the mean length of the newly laid eggs is the same as that of those ready to hatch, 1.68 mm. This would indicate that the egg enlarges transversely during the development of the embryo.

While eggs have been collected in the field as early as June 29, deposition continues throughout the flight period. The majority are laid in turf between  $4\frac{1}{2}$  and 6 inches below the surface. Some have been found at a depth of only  $1\frac{1}{2}$  inches but none below a depth of 6 inches. The maximum number of eggs laid by a single female beetle under insectary conditions was 29 and the minimum 3. A total of 362 eggs was laid by 32 females, the average being between 11 and 12 per female. Females, collected in the field from pupal skins on July 6 and placed with males on July 7, deposited eggs on July 8. The majority of the eggs in the insectary hatched in 20 to 22 days. Just before the larva hatches, the mandibles and portions of the head capsule, which have become darkened in color, are visible through the chorion.

## THE LARVA

The larva passes through three instars before attaining full growth. The mean dorsal length of twenty-one 1-day-old larvae was  $5.08 \pm 0.05$  mm. The mean cranial width of 20 first-instar larvae was  $1.57 \pm 0.01$  mm. The mean cranial width of 15 second-instar larvae was  $2.32 \pm 0.01$  mm. Full grown third-instar larvae differ somewhat in size. The mean length of 27 specimens was  $22.7 \pm 0.12$  mm., individuals ranging from 20 to 24 mm. The mean cranial width of 44 specimens was  $4.12 \pm 0.01$  mm.

The head of the full-grown larva is yellowish brown in color, and the body is white. The anal slit is transverse and arcuate. The raster has a sparse group of coarse, long, hooked, brownish spines, which become larger toward the anal slit. Figures of the full-grown larva, together with structural characteristics of the epipharynx, mandibles, maxillae, and prothoracic and metathoracic legs are given in figure 1.<sup>5</sup>

<sup>5</sup> The figures of the pupae and the epipharynx of *C. immaculata* were prepared by the author; all others are by Elizabeth Kaston.

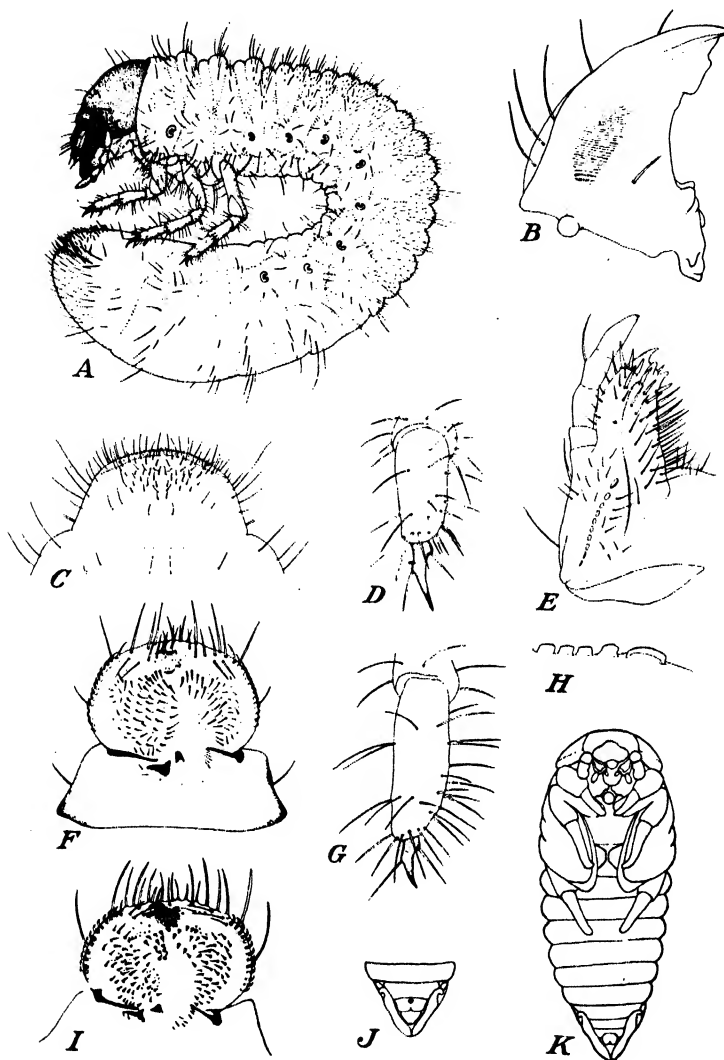


FIGURE 1.—Larva and pupa of *Cyclocephala borealis*: A, Larva entire; B, right mandible of the larva, showing area of striation; C, ventral aspect of the anal segment of the larva; D, prothoracic leg of the larva; E, cephalic aspect of the left maxilla of the larva, showing one very large, seven median, and two small stridulating teeth; F, epipharynx of the larva; G, metathoracic leg of the larva; H, the five anterior stridulating teeth of the left maxilla of the larva; I, epipharynx of *C. immaculata*; J, ventral view of the last three segments of the female pupa; K, ventral view of the male pupa.

Morphologically the larvae of *Cyclocephala borealis* Arrow and *C. immaculata* Oliv.<sup>6</sup> are very similar, but the haptomeri of the two

<sup>6</sup> Appreciation is hereby acknowledged to Dr. P. O. Ritcher for the receipt of many fine larval specimens of *C. immaculata* from Kentucky.



species are somewhat different in structure. A figure of the epipharynx of *immaculata* is shown in figure 1, K. The haptomerum of *borealis* has a definite single, large, sclerotized, rounded plate and straight apex, with a deep notch to the left of the center of the zygum. The darkened chitinated anterior edge on the right lobe is about four times as broad as it is deep. The breadth of the left lobe likewise is about twice its depth. In *immaculata* the rounded, sclerotized plate does not terminate as definitely as that of *borealis*, and the notch is more centrally located but rather indistinct. The lobes are more nearly equal in size, with the dark, chitinated apical edge of the right lobe more than two times as broad as it is deep, while the breadth and depth of the left lobe are about equal. The dark, chitinated apices of the lobes are more distinct than in *borealis*.

Feeding begins almost immediately after hatching, and larvae less than 1 day old are discolored by food in the alimentary tract. The larvae feed on organic matter and on the roots of plants, in Connecticut primarily on the roots of turf.

Damage to turf usually occurs in September or October and in the spring. Three diggings in different localities where such injury was evident yielded, respectively, 33, 48, and 64 third-instar larvae to 1 square foot. Third-instar larvae were observed in the field on September 7, and it is evident from field observations that the larvae are in this instar when they go into hibernation.

The larvae react to changes in soil temperature in a manner similar to that of other scarabacid larvae, descending at the onset of cold weather in the fall and ascending in the spring. Larvae in individual cases will descend to a depth of 18 inches to hibernate. In 1939 the descent began early in November, when the larvae were found distributed at depths ranging from 2 to 14 inches, as shown in table 1. One digging made on April 12, 1938, before the upward movement of the grubs took place, revealed 11 larvae in the upper 3 inches of soil, 19 larvae at a depth of from 3 to 9 inches, and 1 larva more than 9 inches deep. Winter mortality may be heavy; in one digging 30 of 61 grubs found were dead. At a later date in diggings made elsewhere, many dead grubs were noted at hibernation depths.

TABLE 1.—Depth to which larvae of *Cyclocephala borealis* descend to hibernate, 1939

Date	Number of larvae found at depth indicated (inches)													
	2	3	4	5	6	7	8	9	10	11	12	13	14	
Nov. 9	6	5	0	4	3	4	4	2	2	2	1	0	0	
Nov. 14	0	2	4	4	9	2	6	3	5	0	2	0	1	

#### THE PUPA

The pupa when newly transformed is creamy white in color, but it gradually turns a reddish brown. The mean dorsal length of 16 preserved pupae was  $16.78 \pm 0.18$  mm. Figure 2 illustrates the differences between the sexes. The male genital segment (ninth abdominal) bears a pair of conspicuous lobes. In the female this segment is partially divided by a short median suture, anterior to which is situated the gonotreme. The sex of the older pupae may also be determined by observing the sexual differences of the tibial spurs through the cuticle of the prothoracic legs.

The pupal period occurs during June. On June 8, 1939, a digging adjacent to the shore of Long Island Sound yielded 25 larvae, 51 prepupae, and 41 pupae. A second digging was made at the same place on June 19, 1939, and 3 larvae, 2 prepupae, and 36 pupae were

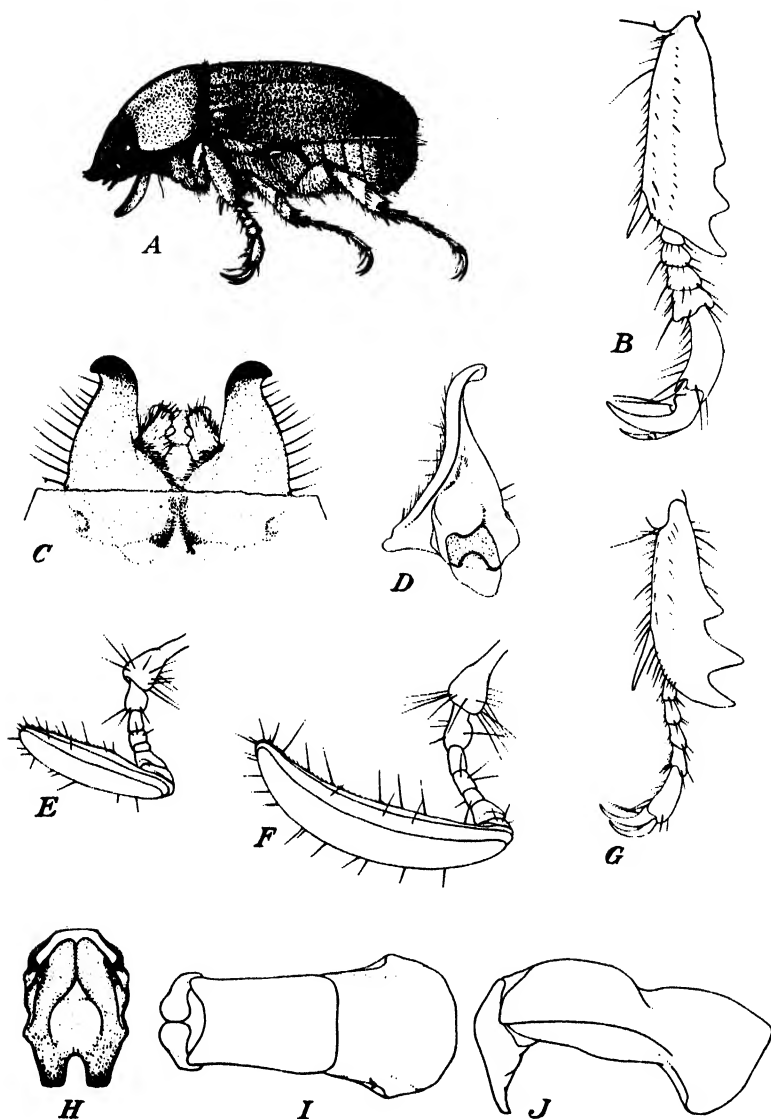


FIGURE 2.—*Cyclocephala borealis*: A, Adult male; B, prothoracic leg of the adult male; C, dorsal aspect of the mandibles of the adult male in situ; D, mesal aspect of the right mandible of the adult; E, female antenna; F, male antenna on same scale as E; G, prothoracic leg of the adult female on same scale as B; H, end view of the male genitalia; I, dorsal view of the male genitalia; J, lateral view of the male genitalia.

found. An examination of larvae placed in a 24-inch glazed tile the preceding fall was made on June 9, 1939, and 42 larvae and 2 prepupae were found. About 75 larvae were used for rearing purposes at room temperature and also at 78° F. The prepupal period was 4 to 5 days in length. The length of the pupal period varied from 11 to 16 days, but the largest number emerged as adults on the fourteenth and fifteenth days.

#### THE ADULT

The adult is chestnut brown in color and covered with fine hairs, the male being slightly larger and somewhat darker than the female. The mean dorsal length of 22 adult males collected from pupal skins was  $11.84 \pm 0.10$  mm. and the breadth  $6.76 \pm 0.06$  mm. The mean dorsal length of 22 females collected from pupal skins was  $11.03 \pm 0.07$  mm., and the breadth  $6.66 \pm 0.04$  mm. The males are distinguished by their larger tarsal claws, shorter and narrower tibial spurs, and longer antennal lamellae. In figure 2 are drawings of a male, the prothoracic legs and antennae of the male and female, and the male genitalia. The body of *Cyclocephala borealis*, (3, pp. 989-990), is pubescent above, and the antennal club of the male is longer than all the other antennal joints together. *C. immaculata* is smooth above, and the antennal club of the male is shorter than all the other antennal joints together.

The adults are nocturnal in habit and remain in the soil during the day. Careful observation during the evening when the adults were active and again during daylight hours failed to indicate feeding of any kind. The alimentary tract was empty and atrophied in all specimens examined. The mandibles are fully developed but apparently unfitted for chewing, for they are anteriorly excurvate rather than incurvate (fig. 1, C).

Just about dusk, or half an hour before dark, on warm, humid evenings, adults may be observed emerging from the soil and climbing to the tips of the grass in lawns. Occasionally one will take wing and fly rapidly just above the ground. As the darkness deepens, more and more beetles emerge and greater activity is observed. From investigations made during the last 2 years when adults were present, it appears that males are predominant in activity and flight. They usually fly from 1 to 2 feet above the ground in search of females. Some, however, will fly somewhat higher and dart away in the dark or toward a light to which they are attracted. When the insect takes to flight, the wing vibrations have a characteristic sound. Flight and general activity cease before midnight. On cool evenings, flight is lessened and the adults are present only for a brief period.

The female beetles emerge and rest on the surface of the soil, if it is void of grass, or in the turf areas ascend to the tips of the grass. In a moment they are captured by a male, and copulation takes place directly. The males clasp the females along the edge of the elytra behind the legs with their tarsal claws. The females often move about, carrying the males. If disturbed, when single or paired, the adults invariably immediately seek shelter in the grass or loose soil.

In the rearing experiments, adults were removed directly from the soil in their pupal cases, they mated the next day, and eggs were obtained the third day. When individuals were placed together upon collection from their pupal cases in the field, mating immediately took place, and the following day eggs were obtained.

In 1938 the first adults were captured around the lights and windows of a private garage on the evening of June 24. The largest number were captured about July 5, and the last on July 25.

The life cycle requires 1 year; and the stages, briefly summarized, are as follows: Egg, 20 to 22 days in June and July; larva, 10 to 11 months from July to June; prepupa, 4 to 5 days in June; pupa, 11 to 16 days; and adult, 5 to 25 days in June and July. As the transformations do not all occur at the same time, there is overlapping of the stages.

#### CONTROL

The larvae are to some extent parasitized by a species of *Tiphia* as yet undetermined. Of 1,000 larvae examined, 25 were found to be so parasitized.

Applications of lead arsenate, made in 1938 under the author's direction in Westport and Greenwich at the rate of 20 and 30 pounds, respectively, to 1,000 square feet of lawn, gave excellent control. Neiswander (8) reports that he obtained approximately 70 percent control in 1 month, using lead arsenate at the rate of 10 pounds to 1,000 square feet. He also reported good control by applications of carbon bisulfide emulsion diluted with water.

Light traps were used by the author in Westport to capture adult beetles. At first the traps were suspended on standards 3 to 4 feet above the ground, but after the flight habits of the beetles were observed, the traps were set directly on the ground and an increased number were captured. Homemade traps, consisting of an ice-box drip pan containing water with a layer of kerosene oil one-fourth of an inch in depth, four pieces of window glass 9 by 12 inches placed vertically to serve as baffles, and a 100- or 150-watt frosted bulb suspended directly above the baffles, were very successful. A 300-watt daylight bulb was tried in the area of immediate infestation but was not so successful as the lower-powered bulbs.

Three such traps were used in one area of about 3 acres and a total of 18,967 beetles were captured. Of 9,029 adults captured in two of the traps and determined for sex, 8,996, or 99.63 percent, were males and 33, or 0.37 percent, were females. The traps were in operation from June 25 to July 29. Thirteen of the females were captured on the night of July 5 when the maximum catch was made, and the remaining 20 were taken on 6 other nights. This result was similar to that obtained by Neiswander (8). Light traps are not recommended for control. However, if the beetles should be attracted by lights to residences or recreation centers and become a nuisance, light traps might be useful.

#### SUMMARY

*Cyclocephala borealis* Arrow was first found in Connecticut in 1931, in the town of Westport, where larvae were destroying lawn turf. It has since been found in several other towns in the southwestern part of the State, and during the past 9 years has injured or destroyed many acres of fine turf on lawns and golf courses.

The adults are chestnut brown in color and are present for about 1 month, beginning about June 25. They are nocturnal in habit, emerging from the soil about dusk, and are active during the early hours of the evening. The males are the more active.

Oviposition begins the first or second day after the adults first emerge, and the eggs hatch in about 3 weeks. The larvae pass through three instars, usually entering the third instar in early September. A field population of 64 larvae per square foot was recorded in one lawn. The larvae feed upon the roots of grass, and turf injury may first appear in September. The larvae ascend and descend in the soil with the rise and fall of the soil temperatures in the spring and fall. Hibernation occurs in the larval stage during the winter at depths varying from 2 to 18 inches, with the majority of the larvae at depths between 3 and 9 inches. The larvae complete their growth by late May or early June. The insect spends from 4 to 5 days as a prepupa in early June. It remains in the pupal stage some 2 weeks, emerging as an adult about the third week in June. The adults have not been observed to feed, and their mouth parts are unsuited for feeding.

Both male and female beetles are attracted to lights. However, light traps capture a great predominance of males and are not recommended for control. The larvae may be controlled by treating the turf with lead arsenate or carbon bisulfide emulsion diluted with water.

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# GERMINATION REDUCTION AND RADICLE DECAY OF CONIFERS CAUSED BY CERTAIN FUNGI<sup>1</sup>

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## INTRODUCTION

Increased attention has recently been directed to the frequent recurrence of failures of emergence in coniferous nursery beds. Sometimes the failures have been severe, completely covering large blocks of beds; at other times they have been spotty over several beds. It has long been known from observation (2, 3, 4)<sup>2</sup> that decreases in emergence of pine seedlings may be indicative of damping-off epidemics, and it has been shown by inoculations with damping-off organisms that emergence failures can be produced in steamed soil and that failures due to these organisms can be controlled in the nurseries by the same soil-disinfectant treatments that control damping-off.

There is reason to suspect, however, that seeds are decayed by organisms other than those known to cause damping-off. Some rather serious failures have occurred in beds sown with seed from lots that had germinated well in other trials. Numerous failures have been observed where soil cover and moisture were apparently correct and damping-off fungi were not appreciably active. Failures have been most frequent in cold, wet soil where such emergence as did occur was slow, and nonsprouted seeds removed from such beds have often been found to be rotted.

In routine seed investigations a cutting test is made on each lot of seed collected. One hundred seeds are cracked open and classified, according to the appearance of the embryo, into good seed, rancid seed, and "blind" seed, that is, seeds that have not been properly filled. The cutting test is indicative of the germination potential of the lot. Frequently, however, the percentage of germination in the field is lower than the percentage of seed classified as good. Some of the seeds, therefore, that appear viable may be immature or in a weakened condition and thus may be more susceptible to attack by organisms that may or may not usually be parasitic. It is known that the micropyle of the bean is occasionally glutted with bacteria. While coniferous seeds have hard coats and no similar open chamber for the carrying of organisms, there is some opportunity for organisms to enter the seeds before they fully mature in the cone, as a result of injury in the extraction process or because of improper storage. If decay occurs in a treated soil, therefore, it is conceivable that the pathogen was carried into the soil in or on the seed itself.

<sup>1</sup> Received for publication October 1, 1940. The work reported herein was supported chiefly by funds of the Civilian Conservation Corps.

<sup>2</sup> Italic numbers in parentheses refer to Literature Cited, p. 94.

## PREVIOUS INVESTIGATIONS

Many investigations have been reported on the causes and control of damping-off in both deciduous and coniferous seedbeds. Active work on the control of damping-off of conifers is still in progress. The term "germination loss" has been used for reduced emergence resulting from seed decay or preemergence damping-off, or both, since in ordinary seedbed practice the seed is covered and the two types of loss cannot be distinguished. Hartley (2, 3, 4) attributed this loss in coniferous seedbeds to species of *Pythium*, *Rhizoctonia*, and *Fusarium*, on the basis of successful inoculations on steamed soil with representatives of all three genera, including *P. aphanidermatum* under the name of *Rheosporangium*. *Fusarium* spp. on the whole indicated less ability to cause preemergence than postemergence losses. For *Botrytis cinerea* in a preliminary test the reverse appeared true.

Rathbun-Gravatt (5), by inoculations under direct observation, demonstrated the ability of a number of strains of *Pythium*, *Rhizoctonia*, and *Fusarium* to reduce sprouting of coniferous seed. She found that, in general, the fungi that decreased sprouting were the ones that were able to decay radicles.

Fourteen species of fungi "arising from the seed of *Pinus sylvestris*" were determined by Garbowski (1). His methods of sterilization and isolation are not known, however. The germination of *Pinus sylvestris* was greatly reduced by contaminations of *Pyronema omphalodes* and one species of *Botrytis*.

Vanin (8) and Vanin and Kotchkin (9) studied the fungi associated with the seeds and seedlings of tree species. They reported inhibited germination from widespread contaminations of *Penicillium*, *Aspergillus*, *Mucor*, and *Rhizopus*. They grouped seed fungi into three classes: (1) Those that damage seeds; (2) those present in or on seeds but not causing diseases until after germination (damping-off); (3) those not harmful to either seed or seedling.

Ten Houten (7) isolated many fungi from conifer seeds or seedlings; part of the isolations from seed (which ones are not indicated) had been preceded by surface sterilization. Inoculations with more than 50 of these and some others were made on 24 surface-sterilized seeds each of Scotch and Austrian pine in tube cultures, half of which were on filter paper and half on sterilized soil. In his filter-paper cultures (and in the soil tubes also) he had the seed under direct observation, and germination reduction is to be taken more as an indication of seed decay than of preemergence damping-off. So far as inferences can be drawn from so few seeds, there was fairly definite reduction of sprouting by one of the two *Rhizoctonia* lines, by *Botrytis cinerea* and an unidentified *Botrytis*, and very likely some reduction by *Pythium debaryanum* (which the author is understood to regard as including *P. ultimum*), *Phytophthora fagi*, and *Coniothyrium pityophilum*. For the remaining fungi, apparent increases in germination associated with the inoculation were as frequent and as large as the decreases except for the presumably accidental case of *Pyronema confluens* on Scotch pine. Except for the *Pyronema*, all of these fungi that appeared to reduce germination also attacked seedlings, though the *Phytophthora* was relatively weak. There thus appeared, as in Rathbun-Gravatt's work, to be considerable correlation between ability to attack seed and to attack seedlings. However, a number of *Fusarium* species and

related fungi included in the test, and also other species of *Pythium* and another line of *Rhizoctonia*, attacked seedlings without any material reduction in germination.

*Pinus pinea* cones were found by Sibilia (6) to contain discolored, powdery seeds in some of which the kernel was still sound but in others it was blackened or completely destroyed, its place being taken by a grayish mycelial mat. The fungus was tentatively identified as *Alternaria tenuis*. If this fungus had not penetrated the seed before planting, germination was normal.

Busteed<sup>3</sup> reported the decay of *Pinus nigra* seed by *Physalospora obtusa* in nursery beds. The fungus was found fruiting on the cones and in cone inoculation experiments; ovules were destroyed, and the number of seeds produced was markedly decreased.

Since the experiments of Rathbun-Gravatt had been conducted at room temperatures, the purpose of the present investigation was to study the relation of a somewhat different array of organisms and lower temperature to germination reduction and seed decay. Many of the organisms used were isolated from fresh and old seeds.

## ORGANISMS ISOLATED FROM SEEDS

### MATERIAL AND METHODS

Organisms were isolated from fresh coniferous seeds, from seeds from nursery beds where germination was a failure and from stratified seeds. These last were stratified in flats in a nursery soil; a greenhouse potting soil, and a pure quartz sand, all maintained, from sowing to completion of germination, alternately at 40° and 50° F. during each successive 3-day period. Neither seeds nor substrata were sterilized. Isolations were made at weekly intervals until the stratified seeds started to germinate. The seeds were surface-sterilized with a 1-percent solution of chloramine for 20 minutes, or with  $\frac{1}{1000}$ ,  $\frac{1}{500}$ , and  $\frac{1}{250}$  solutions of bichloride of mercury alone or mixed with 95-percent alcohol, the treatment ranging from 8 to 20 minutes. A saturated solution of bromine water diluted to  $\frac{1}{10}$  was also tested, but it proved less satisfactory than the bichloride of mercury and alcohol solution. After sterilization each lot of seed was washed three times with sterile distilled water. The seed was cut open with a flamed instrument. Portions from the inside of the coat, the surface of the endosperm, inside the endosperm, and the embryo were transferred to corn meal, malt, or potato-dextrose agar. Surface-sterilized whole seeds were placed on agar slants as a check on the effectiveness of the sterilization.

### RESULTS

#### FRESH SEEDS

Organisms were found in only about 10 percent of the cultures made from fresh seeds of *Pinus banksiana* Lamb., *P. ponderosa* Dougl., and *P. resinosa* Ait., but enough fungi were present to explain spotty decay in nursery beds and the complete loss of whole beds planted with some lots of seed that were examined. *Aspergillus*, bacteria, *Cladosporium*, *Fusarium* spp., *Mucor*, *Penicillium*, *Rhizopus*, *Trichoderma*, and several unidentified fungi were isolated. Bacteria and *Fusarium* spp. were isolated most frequently.

<sup>3</sup> BUSTEED, ROBERT C. THE PATHOGENICITY OF *PHYSALOSPORA ORTUSA* ON *PINUS NIGRA*. [Unpublished doctor's thesis. Copy on file Ind. Univ. Lib., Bloomington, Ind.]



Substratum and species		Organisms isolated (number of times)											
		<i>Aspergillus</i>	Bacteria	<i>Chaetomium glo- bosum</i> Kze.	<i>Fusarium moni- forme</i> Shield	<i>Fusarium</i>	<i>Mucor</i>	<i>Penicillium</i>	<i>Rhizopus</i>	<i>Thielaria</i>	<i>Trichoderma</i>	Unidentified	Total
Quartz sand:													
<i>Pinus ponderosa</i> Dougl	Good	0	1	1	0	1	0	0	0	0	0	4	7
<i>P. resinosa</i> Alt.	do	0	1	0	0	0	0	0	0	0	0	1	2
<i>P. strobus</i> L.	do	0	3	0	0	0	0	0	0	0	1	3	7
<i>P. caribaea</i> Morelet	do	0	1	0	1	0	0	0	0	0	0	2	3
Do	Fair	0	2	1	0	0	0	0	0	0	0	1	3
Do	Poor	0	1	0	0	0	0	0	0	0	0	0	1
<i>P. taeda</i> L.	Good	0	2	0	0	0	0	0	0	0	0	0	2
Do	Fair	0	1	0	1	0	0	0	0	0	0	0	2
Do	Poor	0	1	1	0	0	0	0	0	0	0	1	2
<i>P. echinata</i> Mill	Good	0	1	1	0	0	0	0	0	0	0	0	2
Do	Fair	0	0	0	0	0	0	0	0	0	0	1	1
Do	Poor	0	1	0	0	0	0	1	0	0	0	0	2
Total		0	15	1	2	1	0	1	0	0	1	12	33

TABLE 1.—Number of times the indicated organisms were isolated from stratified coniferous seeds—Continued

Substratum and species	Quality or condition of seed	Organisms isolated (number of times)											
		<i>Aspergillus</i>	Bacteria	<i>Chaetomium glo- bosum</i> Kze.	<i>Fusarium monil- iforme</i> Sheld.	<i>Fusarium</i>	<i>Mucor</i>	<i>Penicillium</i>	<i>Rhizopus</i>	<i>Thelaria</i>	<i>Trichoderma</i>	Unidentified	Total
Greenhouse soil:													
<i>P. ponderosa</i>	Good	0	2	0	0	2	0	0	0	0	0	4	8
<i>P. resinosa</i>	do	0	5	0	0	0	0	0	0	0	0	2	7
<i>P. strobus</i>	do	0	3	0	0	0	0	1	1	0	0	2	7
<i>P. caribaea</i>	do	0	0	0	0	0	0	0	2	0	0	0	2
Do	Fair	0	1	0	0	0	1	0	0	0	0	1	3
Do	Poor	0	1	0	0	0	0	0	1	0	1	0	3
<i>P. taeda</i>	Good	0	2	0	0	0	0	0	0	0	0	2	4
Do	Fair	0	1	0	0	1	0	0	1	0	0	1	4
Do	Poor	0	1	0	0	0	0	0	0	1	0	1	3
<i>P. echinata</i>	Good	0	2	0	0	0	0	0	2	0	1	0	5
Do	Fair	0	2	0	0	0	0	0	1	0	0	2	5
Do	Poor	0	2	0	0	0	0	0	1	0	0	1	4
Total		0	22	0	0	3	1	1	9	1	2	16	55
Nursery soil:													
<i>P. ponderosa</i>	Good	1	2	0	0	2	0	0	0	0	0	5	10
<i>P. resinosa</i>	do	1	0	0	0	0	0	0	1	0	0	0	2
<i>P. strobus</i>	do	0	5	0	0	0	1	0	2	0	0	6	14
<i>P. caribaea</i>	do	0	1	0	0	1	0	1	0	0	0	1	4
Do	Fair	0	1	0	0	0	0	0	1	0	0	1	3
Do	Poor	0	1	0	0	0	0	0	1	0	0	0	2
<i>P. taeda</i>	Good	0	1	0	0	1	0	0	1	0	0	1	4
Do	Fair	0	0	0	0	0	0	0	1	0	1	2	4
Do	Poor	1	0	0	0	1	0	0	0	0	0	0	2
<i>P. echinata</i>	Good	0	2	0	0	0	0	0	1	0	0	1	4
Do	Fair	0	2	0	0	1	0	0	1	0	0	1	5
Do	Poor	0	0	0	0	0	0	0	1	0	1	1	3
Total		3	15	0	0	6	1	1	10	0	2	19	57

## INOCULATION EXPERIMENTS

## MATERIAL AND METHODS

The cultures of *Alternaria brassicae* var. *microspora* P. Brun., *Pythium* spp., *Rhizoctonia* sp., and *Sphaeropsis ellisii* Sacc. that were used in the inoculation experiments were obtained from the stock cultures of the Division of Forest Pathology. The *Botrytis* culture was obtained from Dr. Charles Brooks, of the Bureau of Plant Industry, and had been isolated from blackberry fruit. The rest of the fungi used (see table 2) were isolated from pine seeds.

For these experiments, 30 cc. of Difco corn-meal agar, one-half concentration of the formula stated on the bottle, plus 15 gm. of agar was poured into Petri dishes. The plates were then inoculated with the fungi to be tested. The growth of the fungi was inhibited to such a degree by the low temperatures used that it was necessary to keep the plates at room temperature for a few days to obtain a good coverage of the agar surface. The plates were then seeded with various pine species and stored at low temperatures as shown in the footnotes of table 2. Experiment 1 was conducted at the lowest temperatures and experiments 4 and 5 at the highest temperatures. When testing organisms producing heavy mycelial mats, two or three small rectangles, approximating 2 inches long and 0.5 inch wide, were cut from the agar of the fungus culture in each plate and the seed

was placed on the glass along the edges of and in contact with the agar, since otherwise the mat would have kept the seed from taking up moisture.

The fungi were reisolated from radicles showing decay.

### RESULTS

The average radicle decay in the different types of experiments with each species is given in table 2.

TABLE 2.—Germination reduction and radicle decay of pines in agar plates inoculated with various fungi at different temperatures

Experiment, temperature, and inoculating fungus	Germination in uninoculated units and reduction (—) or increase (+) in inoculated units (percentage of total seeds)				Radicle decay (percentage of total radicles)			
	<i>Pinus resinosa</i> <sup>1</sup>	<i>Pinus ponderosa</i> <sup>2</sup>	<i>Pinus banksiana</i> <sup>3</sup>	Weighted average	<i>Pinus resinosa</i>	<i>Pinus ponderosa</i>	<i>Pinus banksiana</i>	Weighted average
Experiment 1 (32° and 50° F.): <sup>4</sup>								
<i>Botrytis cinerea</i> .....	0				9			
<i>Sphaeropsis ellisi</i> Sacc.....	-10				29			
Uninoculated.....	87				0			
Experiments 2 and 3 (40°, 50°, and 60° F.): <sup>4</sup>								
Unidentified (No. 114).....	-33	-27		-31	8	26		12
<i>Fusarium</i> sp. (No. 132a).....	-33	-16		-20	1	9		3
<i>Botrytis cinerea</i> .....	-27	-27		-27	8	7		8
Plaster mold.....	-17	-38		-22	2	0		2
<i>Fusarium conglutinans</i> Wr.....	-15	-22		-17	31	17		28
<i>Penicillium</i> sp.....	-20	-2		-16	0	13		4
Phycomycete (unidentified).....	-15	-16		-15	6	13		8
<i>Cylindrocylindrium</i> sp.....	-9	-31		-14	5	0		4
<i>Aspergillus niger</i> Tiegh.....	-9	-18		-11	16	16		16
<i>Pestalotia</i> sp.....	-7	-25		-11	4	0		3
<i>Sphaeropsis ellisi</i> .....	-11	-11		-11	11	27		15
Unidentified (No. 19).....	-3	-29		-9	2	0		1
<i>Rhizopus oryzae</i> Went (No. 104) <sup>5</sup> .....	-7	-18		-9	0	0		0
<i>Rhizoctonia</i> sp.....	-3	-27		-8	4	7		4
<i>Alternaria brassicae</i> var. <i>microspora</i> P. Brun.....	-3	-18		-6	0	0		0
<i>Verticillium</i> .....	-3	-11		-4	1	3		1
Average of inoculated.....	-13	-21		-16	6	9		7
Uninoculated.....	90	87		89	0	0		0
Experiments 4 and 5 (60° and 77° F.): <sup>1</sup>								
<i>Aspergillus niger</i> .....	-42	-40	-57	-49	0	0	0	0
<i>Pythium ultimum</i> Trow.....	-27	-13	-48	-33	82	95	100	93
<i>Chaetomium globosum</i> Kze.....	-25	-29	-20	-24	0	0	0	0
<i>Pythium delarvianum</i> Hesse.....	-18	0	-36	-21	78	67	95	82
Unidentified (No. 178).....	-7	-2	-33	-14	62	77	100	82
<i>Rhizopus</i> sp. <sup>6</sup> .....	-22	-2	-7	-10	0	0	0	0
<i>Rhizopus oryzae</i> (No. 199) <sup>6</sup> .....	-18	+2	-11	-9	0	0	0	0
<i>Rhizopus circinans</i> Tiegh. <sup>6</sup> .....	-18	-2	-7	-9	0	0	0	0
<i>Mucor</i> sp. <sup>6</sup> .....	-13	-9	-4	-8	0	0	0	0
<i>Thielavia</i> sp.....	-16	+4	-9	-7	0	0	0	0
<i>Fusarium</i> sp. (No. 38).....	-5	-4	-8	-6	18	32	19	22
<i>Penicillium</i> sp.....	-18	+2	-4	-6	0	0	0	0
Unidentified (No. 64).....	-9	-2	-8	-7	3	0	3	2
<i>Fusarium</i> sp. (No. 132a).....	-7	-11	+3	-4	19	18	36	28
Unidentified (No. 145).....	-13	+9	-5	-4	27	19	58	40
Unidentified (No. 179).....	-9	+13	-5	-1	0	12	13	9
Unidentified (No. 180).....	-9	+18	-2	+1	0	0	0	0
Average of inoculated.....	-16	-4	-15	-12	18	19	25	22
Uninoculated.....	89	60	91	82	0	0	0	0

<sup>1</sup> 50 seeds per plate in experiments 1 to 3, and 15 seeds per plate in experiments 4 and 5. Plates in triplicate in each experiment.

<sup>2</sup> 15 seeds per plate. Plates in triplicate in each experiment.

<sup>3</sup> 25 seeds per plate. Plates in triplicate in each experiment.

<sup>4</sup> Seeds sterilized for 20 minutes in 1-percent chloramine solution. From sowing to completion of germination plates stored alternately at 32° and 50° F. during each successive 3-day period.

<sup>5</sup> Seeds not sterilized. For 4 weeks after sowing plates stored alternately for 1 day at 40° F. and 2 days at 50° and for the next 8 weeks alternately for 1 day at 50° and 2 days at 60°.

<sup>6</sup> Tips soft but not discolored.

<sup>7</sup> Seeds sterilized for 12 minutes in 1/250 HgCl<sub>2</sub> and washed in 3 changes of sterile water. From sowing to completion of germination plates stored alternately for 2 days at 60° F. and 1 day at 77°.

<sup>8</sup> Apparently a different species from *R. oryzae* and *R. circinans*.

Since *Aspergillus*, *Penicillium*, and *Fusarium* No. 132a were the only three species used in all experiments 2 to 5, no detailed comparisons as to the effects of temperature can be made. However, *Aspergillus*, *Chaetomium*, *Mucor*, *Penicillium*, *Thielavia*, and three species of *Rhizopus* did not cause radicle decay of any of the species tested at 60° and 77° F.; *Alternaria* and *Rhizopus oryzae* did not cause radicle decay at the lower temperatures of experiments 2 and 3.

*Aspergillus* decayed radicles of both pines tested, and *Penicillium* decayed radicles of *Pinus ponderosa* but not of *P. resinosa* in experiments 2 and 3.

In experiments 4 and 5 *Pythium debaryanum* and *P. ultimum* caused much radicle decay in all three pines, the only instance known to the writer in which these two species have been directly compared on conifers. *P. debaryanum* was very destructive, but always less so than *P. ultimum* in respect both to radicle decay and germination reduction. In the same experiments the two strains of *Fusarium* used were consistently pathogenic on all species.

In experiments 2 and 3 *Fusarium* No. 132a only slightly affected the radicles of *Pinus resinosa* and caused less radicle decay of *P. ponderosa* than at the higher temperatures, while *Fusarium conglutinans* caused much decay in both species. *Sphaeropsis* caused a moderate amount of loss.

*Botrytis*, an unidentified phycomycete *Rhizoctonia*, and *Verticillium* caused a small amount of radicle decay in experiments 2 and 3. *Cylindrocladium*, an unidentified species (No. 19, tentatively identified as *Macrosporium*), *Pestalozzia*, and plaster mold caused a slight decay of *Pinus resinosa* radicles but did not affect *P. ponderosa* seedlings in these experiments.

Five of the six unidentified fungi tested caused some radicle decay. No. 64 has been tentatively identified as *Fusarium trichothecioides* Wr. and No. 114 as a mixture of *Fusarium oxysporum* Schlecht. and *Penicillium frequentens* Westling.

As shown in table 2, there was a general decrease in the percentage of germination in practically all inoculated units, even when there was no visible radicle or seed decay. This result was unexpected and requires some further consideration. In experiments 4 and 5, although *Aspergillus niger* did not cause radicle decay, it did cause a marked reduction in germination. So also did *Chaetomium globosum*. Except in one instance in experiments 4 and 5, the fusaria reduced germination, although none of the strains were consistent in respect to the severity of the reduction. The three species of *Rhizopus* and *Thielavia* caused a greater reduction in germination with *Pinus resinosa* than with the other two pines. *Sphaeropsis* did not cause a great reduction with either of the pines on which it was tested.

Temperature exerted a marked influence on the rate of germination in these experiments. In the cultures alternating at 32° and 50° F., initiation of germination was delayed for 10 weeks and the plates finally had to be removed to the laboratory before some of the last seeds would germinate. In experiments 2 and 3 germination was slow and spread over a 3-month period. At the highest temperatures it was completed in approximately 30 days. In general, there was a greater germination reduction in the writer's tests than in the tests by Rathbun-Gravatt (5), and the correlation between germination reduction and radicle decay was not so apparent as in her work. The

heavy mycelial mats of some cultures may possibly have reduced germination by reducing the supply of oxygen, and some of the germination reduction may have been caused by a supply of moisture inadequate for both fungus and seed. However, there was evidence in many cases that sufficient moisture persisted throughout the test or that seeds were definitely decayed, indicating that germination reduction was caused by the direct action of the fungus.

There was no significant difference in the susceptibility of the hosts under the conditions of these experiments.

### SUMMARY

The frequent recurrence of germination failures in coniferous nursery beds suggests the need for extensive studies to determine the proper means of handling seeds and to learn more of their possible pathogens.

A series of organisms was cultured from the interior of fresh and stratified seeds and from seeds taken from nursery beds in which germination had failed. The seeds were surface-sterilized with a 1-percent chloramine solution and with various concentrations of bichloride of mercury alone and mixed with 95-percent alcohol. Some of these organisms were used in the inoculation experiments reported. Bacteria and fusaria were isolated most frequently. The temperatures employed in the experiments were rather low.

In all species tested the following organisms caused decay of radicles just emerged from the seed coats: *Botrytis (cinerea?)*, *Fusarium* spp., an unidentified phycomycete, *Pythium debaryanum* Hesse, *P. ultimum* Trow, *Rhizoctonia*, *Sphaeropsis ellisii* Sacc., *Verticillium*, and four unidentified fungi.

The following organisms did not cause a consistent loss of radicles in the various experiments: *Aspergillus niger* Tiegh., *Cylindrocladium*, *Penicillium*, plaster mold, *Pestalozzia*, and two unidentified fungi.

The following fungi failed to attack radicles: *Alternaria brassicae* var. *microspora* P. Brun., *Chaetomium globosum* Kze., *Mucor*, *Rhizopus* sp., *R. circinans* Tiegh., *R. oryzae* Went, *Thielavia*, and one unidentified fungus.

There was a general decrease in the percentage of germination in practically all inoculated units, even when there was no visible radicle or seed decay. This reduction in germination was greater than in earlier published tests conducted at room temperatures.

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# THE PERSONAL ELEMENT AND LIGHT AS FACTORS IN THE STUDY OF THE GENUS *FUSARIUM*<sup>1</sup>

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## INTRODUCTION

Results previously published<sup>2</sup> suggested that some of the difficulty experienced by different investigators in identifying species of *Fusarium* was due, in part at least, to a lack of uniformly controlled environmental conditions. Among other things it was shown (1) that cultures grown in daylight or in artificial light produced significantly longer conidia than those grown in the dark; (2) that the ratio of macroconidia to microconidia was greatest in the light and least in the dark; (3) that sporodochia and pionnotes were more copious in the light than in the dark; (4) that vegetative growth was most abundant in the dark; (5) that exposure of cultures to light for the first few days after inoculation was more influential in producing macroconidia and conidia of greatest length than similar exposure during the later period of growth; and (6) that statistically significant differences in distribution of conidia of various lengths were recorded when different investigators measured conidia from the same microscopic preparation.

The results referred to above suggested the desirability of further investigations of the inconsistencies and variations that may be expected from the use of the present cultural methods. In addition to those conditions already mentioned that influence the behavior of different species of *Fusarium* in culture, it is important to acquire some knowledge of (1) the homogeneity among different tubes of cultures of the same organisms grown under supposedly identical conditions on the same medium, and the variation among different microscopic mounts from the same spore suspension; (2) the variation in distribution of different lengths of three-, four-, and five-septate conidia of different species grown in light and in darkness on different culture media; (3) the influence of light and darkness on the septations of the conidia; (4) the influence of the substrata on septation of the conidia; (5) the number of spores that should be measured in order to obtain a dependable value of mean length; and (6) the personal element of error due to different investigators.

## MATERIAL AND METHODS

### SPECIES OF *FUSARIUM*

Two species of *Fusarium* (*F. martii* var. *pisi* F. R. Jones and *F. bulbigenum* var. *batatas* Wr.) have been employed in this study. Inasmuch as these investigations were practically an extension of

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<sup>2</sup> HARTER, L. L. INFLUENCE OF LIGHT ON THE LENGTH OF THE CONIDIA IN CERTAIN SPECIES OF *FUSARIUM*. Amer. Jour. Bot. 26: 234-243. 1939.



previous work, use of the same species was believed to be desirable. Both were known to produce pionnotes and sporodochia freely in certain media and to respond readily to different degrees of illumination.

*Fusarium martii* var. *pisi*, a representative of the section Martiella, is the cause of root rot of pea (*Pisum sativum* L.) and produces green pionnotes or sporodochia on cooked potatoes, 2-percent potato-dextrose agar, cooked green bean pods, corn-meal agar, and certain other media. By frequent transfers to suitable media, it has been maintained in "high culture" for several years—a condition highly essential for investigations of this type.

*Fusarium bulbigenum* var. *batatas*, a representative of the section Elegans, is a vascular parasite and the cause of the wilt or stem rot of sweetpotatoes (*Ipomoea batatas* (L.) Lam.). It produces copious pionnotes and sporodochia on corn-meal agar, 2-percent potato-dextrose agar, and other substrata when first isolated. This species was more difficult to maintain in high culture than *Fusarium martii* var. *pisi*.

#### EXPERIMENTAL PROCEDURE

The methods of experimentation were substantially the same as those published in a previous article.<sup>3</sup> Only the methods that differed from those previously described and that were introduced to meet the requirements of specific experiments will be discussed. Solar irradiation was generally employed, although artificial light was used in a few cases.

Cultures grown in the light and in the dark were identical in every respect, except that those culture tubes from which the light was excluded were wrapped in several thicknesses of black paper; the cotton plug was not wrapped. Following inoculation of the media, the tubes were placed on the ledge of a north window, where the temperature varied from 15° to 24° C., or in an artificially lighted incubator. Under the conditions of the experiments, sporodochia began to develop in about 5 to 8 days, and in 10 days pionnotes were frequently present. The cultures appeared to be mature in about 14 days, but the spore counts or measurements were not made until the cultures were 18 days old.

#### RESULTS

##### DIFFERENCES IN RESULTS DUE TO INDIVIDUAL WORKERS

Two investigators familiar with pathological technique and the technique of measuring conidia participated in this experiment. Cultures of *Fusarium martii* var. *pisi* on 2-percent potato-dextrose agar and *F. bulbigenum* var. *batatas* on corn-meal agar were grown in the light for 18 days on the ledge of a north window. At the end of that time, a thin sowing of conidia was made on a microscopic slide and the edges of the cover glass were sealed with paraffin to prevent evaporation of the mounting fluid and movement of the spores. Each participant measured 500 three-septate conidia to the nearest line on the eyepiece micrometer. The participants were instructed to begin at one side of the microscopic preparation and to move the slide in one direction in order not to cover the same field more than once. Each

<sup>3</sup>See footnote 2.

spore measurement was later converted into microns. The spaces on the eyepiece micrometer are equivalent to  $2.5\mu$ . In the tables, there is an interval of  $2.5\mu$  between classes and the number of conidia in each class is recorded.

The 500 measurements of each participant were divided into 10 groups, the first 50 measurements constituting the first group, the second 50 the second group, the third 50 the third group, and so on, which made it possible to calculate statistically the number, in multiples of 50 conidia, that should be measured to obtain mean values that would show no significant difference from means based on somewhat larger numbers.

The frequency distribution of the 10 groups, based on spore length in microns, is shown in tables 1 and 2. The mean length, standard error of the mean, and the coefficient of variation have been calculated from these measurements. Table 1 shows that B recorded more conidia in the  $27.5\mu$  class than A (11:3). On the other hand, A recorded more conidia in the  $50.0\mu$  class than B (12:5). In the case of both A and B, a large percentage of the conidia fell into classes  $37.5\mu$  to  $45.0\mu$ . If the mean of the total number of spore measurements of each participant is compared, a small but inconsequential significant difference is found.

The following conclusions may be drawn from A's data: (1) A comparison with the mean of the 500 spore measurements of the mean of each of the 10 groups of 50 spore measurements shows that none are significantly different; (2) when the mean of the first group of 50 measurements is compared with the mean of each of the other 50-spore populations, it is found that it is not significantly different; (3) if the means of 50, 100, 150, 200, etc., spore measurements are compared with the mean of 500, no significant difference is found.

The following conclusions may be drawn from B's data: (1) A comparison of the mean of 500 spore measurements with the mean of each of the component groups of 50 spore measurements shows that 1 is significantly different; (2) when the means of the other 50-spore populations are compared with the mean of the first 50 spore measurements, 2 are found significantly different; (3) if the means of 50, 100, 150, 200, etc., spore measurements are compared with the mean of 500, 1 (100) is found significantly different. The 100 population was obtained by adding the first and second columns, the 150 by adding the first 3 columns, and the 200 by adding the first 4, etc. It should not be presupposed, however, that the results from a single tube would necessarily be identical with, or even representative of, other cultures of the same organism.

Table 2 reveals about the same facts as table 1. There are some minor significant differences in most cases between the data obtained by A and B. There is a significant difference between the two observers from the first 50 spore measurements. The results of A and B taken separately show a high degree of consistency. No significant difference was found between 50, 100, 150, and 200 spore measurements of either A or B, which indicates that under the conditions of these experiments 50 spore measurements would be about as good as 100, 150, or 200, although the standard error naturally becomes slightly less with the higher number of conidia measured.

TABLE 1.—Frequency distribution of length measurements made by observers A and B of 10 samples of 50 spores each of 3-septate conidia of *Fusarium martii* var. *pisi* grown on 2-percent potato-dextrose agar

Length measurement ( $\mu$ )		Distribution of length measurements as determined by A or B for sample No. —																		Total	
		1		2		3		4		5		6		7		8		9		10	
		A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
27.5	number	2	1	1	1	1	1	1	1	2	1	2	1	2	1	1	1	1	1	1	3
30.0	do.	2	1	2	1	4	3	1	1	1	1	2	1	1	1	2	2	2	1	1	6
32.5	do.	4	3	5	4	6	5	12	6	5	5	2	3	1	5	5	4	5	3	5	25
35.0	do.	6	5	4	5	8	9	10	6	9	10	3	5	2	8	4	4	4	4	3	40
37.5	do.	5	15	13	14	15	11	16	13	11	13	10	5	9	8	8	10	10	8	8	67
40.0	do.	16	8	10	11	11	9	11	10	11	13	10	10	13	10	10	11	13	11	9	119
42.5	do.	12	9	13	11	9	11	7	6	5	6	7	11	10	9	17	8	14	17	15	115
45.0	do.	4	2	1	5	4	6	3	5	1	5	2	5	4	2	6	6	5	3	7	88
47.5	do.	2	1	1	1	1	1	2	1	1	1	1	1	1	2	1	1	1	2	1	25
50.0	do.	1	1	1	1	1	1	2	1	1	1	1	1	1	2	2	1	1	2	2	12
Total	do.	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50	500
Mean length	$\mu$	41.8	41.1	40.8	42.0	41.0	40.0	40.1	39.5	40.4	38.6	40.7	40.5	41.5	38.7	40.5	38.7	40.2	39.8	40.6	39.7
S. E.	$\mu$	$\pm 0.57$	$\pm 0.62$	$\pm 0.59$	$\pm 0.55$	$\pm 0.39$	$\pm 0.76$	$\pm 0.65$	$\pm 0.62$	$\pm 0.62$	$\pm 0.72$	$\pm 0.50$	$\pm 0.75$	$\pm 0.62$	$\pm 0.65$	$\pm 0.63$	$\pm 0.61$	$\pm 0.58$	$\pm 0.56$	$\pm 0.63$	$\pm 0.19$
C. V. %	percent	9.6	10.5	10.1	9.2	10.1	13.3	11.3	11.0	10.7	13.1	8.6	13.0	10.5	11.8	10.9	11.3	10.6	9.7	9.7	11.0

Length measurement ( $\mu$ )		1-2		1-3		1-4		1-5		1-6		1-7		1-8		1-9	
		A		B		A		B		A		B		A		B	
		number	percent	number	percent	number	percent	number	percent	number	percent	number	percent	number	percent	number	percent
Cumulative data:																	
Total	number	100		100		200		250		300		350		400		450	
Mean length	$\mu$	41.3		41.6		40.9		40.8		40.3		40.9		40.9		40.8	
S. E.	$\mu$	$\pm 0.41$		$\pm 0.42$		$\pm 0.30$		$\pm 0.31$		$\pm 0.24$		$\pm 0.28$		$\pm 0.21$		$\pm 0.20$	
C. V. %	percent	9.9		10.1		10.4		10.5		10.2		10.5		13.6		12.0	

: Standard error of mean.

: Coefficient of variation.

TABLE 2.—Frequency distribution of length measurements made by observers A and B of 10 samples of 50 spores each of 4-septate conidia of *Fusarium bulbigenum* var. *batatas* grown on corn-meal agar

Length measurement ( $\mu$ )		Distribution of length measurements as determined by observers A and B for sample No.—																		Total	
		1		2		3		4		5		6		7		8		9		10	
		A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
30.0	number																				
32.5	do																				
35.0	do																				
37.5	do																				
40.0	do																				
42.5	do																				
45.0	do																				
47.5	do																				
50.0	do																				
52.5	do																				
55.0	do																				
Total	do	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50	500	500
Mean length	do	41.4	43.6	41.6	42.0	41.7	42.1	41.9	43.1	41.5	42.8	41.9	42.0	41.9	41.9	42.5	42.4	42.0	43.3	42.8	41.6
S.E.	"	$\pm 0.32$	$\pm 0.39$	$\pm 0.44$	$\pm 0.44$	$\pm 0.37$	$\pm 0.5$	$\pm 0.46$	$\pm 0.45$	$\pm 0.45$	$\pm 0.44$	$\pm 0.52$	$\pm 0.54$	$\pm 0.42$	$\pm 0.41$	$\pm 0.45$	$\pm 0.45$	$\pm 0.42$	$\pm 0.48$	$\pm 0.48$	$\pm 0.14$
C. V.	percent	5.4	6.2	7.4	6.2	8.4	5.8	7.7	7.3	7.6	6.5	7.3	8.7	9.0	7.0	7.4	6.8	7.0	7.3	7.8	7.6

		1-2		1-3		1-4		1-5		1-6		1-7		1-8		1-9	
		A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Cumulative data:																	
Total	number	100	100	150	150	200	200	250	250	300	300	350	350	400	400	450	450
Mean length	"	41.5	42.8	41.6	42.6	41.7	42.7	41.6	42.7	41.6	42.6	41.7	42.5	41.8	42.5	41.8	42.6
S.E.	"	$\pm 0.30$	$\pm 0.27$	$\pm 0.27$	$\pm 0.22$	$\pm 0.23$	$\pm 0.20$	$\pm 0.20$	$\pm 0.18$	$\pm 0.21$	$\pm 0.18$	$\pm 0.18$	$\pm 0.17$	$\pm 0.17$	$\pm 0.15$	$\pm 0.15$	$\pm 0.15$
C. V.	percent	7.2	5.3	7.8	6.3	7.7	6.6	7.7	6.7	8.6	7.2	8.2	7.5	8.1	7.1	9.0	7.5

The mean of 500 conidia measured by B (table 2) and the mean of the same number measured by A differ by an amount that is significant but not important. An examination of the 10 paired means of A and B (table 2) shows that the means of B are higher than those of A, but in table 1 this difference is reversed. One possible explanation for this difference might be suggested. Both observers were instructed to introduce no fractional readings on the eyepiece micrometer. If the length of the conidium extended beyond the midway point of the scale, it was to be recorded as extending to the number above; if it was less than a half, it was to be dropped. When the end of the conidium rested on the halfway point the observer was obliged to use his own judgment, and it is not unlikely that one observer consistently dropped the half fractions and the other read to the next number above.

#### HOMOGENEITY OF CONIDIAL SEPTATION

The ratio or percentage of the number of conidial septations on different culture media has been employed by some investigators in the description of species of *Fusarium*. The value of such ratios as taxonomic characters would seem to depend largely on the constancy with which any given ratio of septations can be expected to occur in spores from cultures grown under the same environment and on the same medium. If a constant ratio could be expected in all cultures thus grown on some standard medium, the variations obtained when the organism was grown on different media would be less important.

To determine the homogeneity of conidial septation in samples taken from different culture tubes, all of the tubes were inoculated from the same spore suspension in water and exposed to the light of a north window. Great care was taken to eliminate as nearly as possible those variations that might result from culturing and mechanical handling. At the end of 18 days of growth the spore masses (pion-notes) in each culture tube were thoroughly mixed. A portion of this mass was then transferred to a few cubic centimeters of water and stirred. From this suspension a thin sowing of conidia was made on a microscopic slide, and the septation of all the spores in a single field of the microscope was counted; the position of the slide was then changed to bring another sample population of conidia into view. This was repeated as often as necessary to accumulate counts on as large a number of individuals as might be required. The number of conidia in a single field of the microscope was intentionally made small so that there would be no confusion in classifying each spore according to the number of septations. Usually the counts were continued until 100 or 200 three-septate conidia were found, the number of two-, four-, and five-septate spores being registered accordingly.

#### HOMOGENEITY OF SEPTATION IN DIFFERENT MOUNTS FROM THE SAME CULTURE

The ratio of three-, four-, and five-septate conidia in 100 spores taken at random was determined for different mounts from one culture tube of *Fusarium martii* var. *psi* and *F. bulbigenum* var. *bataias*. The results are shown in table 3. A similar method of procedure was employed when different tubes were compared.

The  $\chi^2$  values in table 3 indicate that there is no significant deviation from the mean for the 10 mounts in the ratio of three-, four-, and

five-septate conidia for any one of the 10 microscopic preparations of cultures of *Fusarium martii* var. *pisi* grown on either 2-percent potato-dextrose agar or cooked green bean pods. A similar conclusion can be drawn from the data of table 4, which were obtained from the use of a different organism, *F. bulbigenum* var. *batatas*. These data show that a satisfactory homogeneous preparation can be prepared by suspending the conidia in water after thoroughly mixing them in the culture tube.

TABLE 3.—Ratio of 3-, 4-, and 5-septate conidia<sup>1</sup> in 10 microscopic mounts from a spore suspension of a single culture of *Fusarium martii* var. *pisi*, grown on two different culture media

Medium	Septation	Microscopic mount No. —										Total
		1	2	3	4	5	6	7	8	9	10	
2-percent potato-dextrose agar	3	70	75	74	72	71	69	74	74	70	78	727
	4	27	24	25	27	28	30	25	24	30	22	262
	5	3	1	1	1	1	1	1	2	0	0	11
$\chi^2$		3.40	0.35	0.17	0.12	0.25	0.83	0.16	0.94	0.65	1.06	7.94
Cooked green bean pods	3	69	72	75	64	78	74	70	72	71	72	717
	4	28	26	23	34	20	24	29	27	27	27	265
	5	3	2	2	2	2	2	1	1	2	1	18
$\chi^2$		0.26	0.13	0.63	2.97	2.16	0.33	1.0	0.46	0.18	0.46	8.58

<sup>1</sup> 100 conidia observed in each mount.

TABLE 4.—Ratio of 3-, 4-, and 5-septate conidia<sup>1</sup> in 10 microscopic mounts of a spore suspension from each of 2 culture tubes of *Fusarium bulbigenum* var. *batatas* grown on corn-meal agar

Tube No.	Septation	Microscopic mount No.—										Total
		1	2	3	4	5	6	7	8	9	10	
1	3	23	24	20	23	26	19	19	20	23	21	218
	4	20	19	22	19	16	23	24	22	21	21	207
	5	7	7	8	8	8	8	7	8	6	8	75
$\chi^2$		0.34	0.53	0.26	0.24	1.91	0.65	0.92	0.26	0.41	0.36	5.88
2	3	11	14	12	13	7	13	13	18	14	8	123
	4	20	17	22	17	18	20	20	17	17	17	185
	5	19	19	16	20	25	17	17	15	19	25	192
$\chi^2$		0.28	0.37	1.26	0.49	4.17	0.41	0.41	3.68	0.37	3.37	14.81

<sup>1</sup> 100 conidia observed in each mount.

A study of the tables discloses several interesting facts. Table 3 shows a remarkable uniformity in the ratio of the total three-, four-, and five-septate conidia grown on 2-percent potato-dextrose agar and on cooked green bean pods. On the other hand, table 4 shows a somewhat greater difference between tubes that were handled by the same method. Five hundred spores from each tube were classified according to septation, and it was found that the three-septate conidia predominated in tube 1 and the five-septate in tube 2; likewise, the five-septate conidia were fewest in tube 1, the three-septate the fewest in tube 2. The results indicate that, although there seems to be no significant difference between preparations from the same culture, the

various culture tubes may differ very greatly. The differences or the similarities between tube cultures will be more clearly shown in some of the following tables.

Table 5 shows a high degree of homogeneity in the ratio of three-, four-, and five-septate conidia in the two series of cultures. In no case is there any significant deviation from the mean.

TABLE 5.—Ratio of 3-, 4-, and 5-septate conidia in different culture tubes of *Fusarium bulbigenum* var. *batales* grown on corn-meal agar

Series No. <sup>1</sup>	Septation	Tube No.							Total
		1	2	3	4	5	6	7	
1	3	57	52	48	72	39	61		329
	4	71	89	100	100	98	100		558
	5	100	100	94	99	100	81		574
Total		228	241	242	271	237	242		1,461
$\chi^2$		4.80	0.49	1.41	2.62	5.01	3.48		17.81
2	3	39	39	34	39	32	31	36	250
	4	47	37	38	41	37	45	42	287
	5	14	24	28	20	31	24	22	163
Total		100	100	100	100	100	100	100	700
$\chi^2$		4.82	0.71	1.25	0.60	3.33	1.03	0.32	12.07

<sup>1</sup> Series 2 was conducted about 3 months later than series 1; otherwise they were alike.

The ratio of septations of the conidia in some series was very homogeneous; in others it deviated greatly from the mean (tables 6 and 7). Three culture tubes (table 6, series 1) showed  $\chi^2$  values of 12.46, 9.37, and 14.20, respectively, which, with 2 degrees of freedom, is much beyond the 5-percent point. The other 7 tubes in the series were not significantly different from the mean for the 10 tubes. In table 6, the  $\chi^2$  value exceeds the 5-percent point in all cultures in series 2, and in one culture in series 3. Table 7 also shows a lack of homogeneity in the ratio of conidial septation in both series 1 and 2.

TABLE 6.—Ratio of 3-, 4-, and 5-septate conidia in different culture tubes of *Fusarium martii* var. *pisi* grown on 2-percent potato-dextrose agar

Septation	Tube No. (series 1) 1—										Total
	1	2	3	4	5	6	7	8	9	10	
3	88	69	87	85	78	85	91	76	94	68	821
4	12	30	13	14	22	15	9	23	6	32	176
5	0	1	0	1	0	0	0	1	0	0	3
Total	100	100	100	100	100	100	100	100	100	100	1,000
$\chi^2$	2.21	12.46	1.49	0.98	1.31	0.48	4.90	2.27	9.37	14.20	47.66

Septation	Tube No. (series 2) 1—						Total	Tube No. (series 3) 1—						Total
	1	2	3	4	5	6		1	2	3	4	5	6	
3	100	100	100	100	100	100	600	100	100	100	100	100	100	600
4	112	23	22	20	31	81	289	33	49	22	30	33	66	233
5	13	2	4	4	2	5	30	2	3	1	1	1	2	10
Total	225	125	126	124	133	186	919	135	152	123	131	134	168	843
$\chi^2$	21.73	12.07	11.66	13.74	6.06	12.62	76.88	1.00	2.14	6.13	1.75	0.66	12.32	24.60

<sup>1</sup> Series 1, 2, and 3 are identical except that they represent cultures grown at different dates.

TABLE 7.—*Ratio of 3-, 4-, and 5-septate conidia in different culture tubes of Fusarium martii var. pisi grown on cooked green bean pods*

Septation	Tube No. (series 1)---										Total
	1	2	3	4	5	6	7	8	9	10	
3 .....	72	53	68	71	79	80	62	90	72	76	723
4 .....	26	41	27	26	20	20	34	10	22	21	247
5 .....	2	6	5	3	1	0	4	0	6	3	30
Total .....	100	100	100	100	100	100	100	100	100	100	1,000
$\chi^2$ values.	0.40	14.28	2.14	0.03	2.84	1.71	5.3	13.8	3.30	0.74	44.54

Septation	Tube No. (series 2) ---						Total
	1	2	3	4	5	6	
3 .....	100	100	100	95	100	100	595
4 .....	90	33	53	100	54	72	402
5 .....	21	5	12	31	11	29	109
Total .....	211	138	165	226	165	201	1,106
$\chi^2$ values	3.92	20.25	3.35	13.03	3.71	5.39	49.65

<sup>1</sup> Series 2 is the same as series 1, except that the cultures were grown at a different date.

The data indicate that cultures of *Fusarium bulbigenum* var. *bataas* are more uniform (table 5) than cultures of *F. martii* var. *pisi* (tables 6 and 7). *Fusarium bulbigenum* var. *pisi* on corn-meal agar gave no cultures that were significantly different from the mean. Whether or not this would hold true for a large number of cultures and on other media could be determined only by additional trials and experimentation.

Three conclusions may be drawn from these data: (1) That different cultures presumably alike may vary greatly in the ratio of three-, four-, and five-septate conidia; (2) that while three-septate conidia may predominate in one culture, four- or five-septate may predominate in others; and (3) that from a taxonomic standpoint it is unsafe to draw any general and sweeping conclusions based on a few cultures.

#### INFLUENCE OF IRRADIATION ON SEPTATION OF CONIDIA

In a previous publication<sup>4</sup> it was shown that irradiation of the cultures increased the length of the conidia as compared with that of conidia produced in the dark. Its effect on the number of septations was noted at that time, but extensive data were not taken. The methods employed in these later studies were essentially the same as those described in the present paper, and the same two species of *Fusarium* were used, viz, *F. martii* var. *pisi* and *F. bulbigenum* var. *bataas*. *F. martii* var. *pisi* was cultured on 2-percent potato-dextrose agar and cooked green bean pods and grown in a culture chamber at 25° C. The cultures grown in the light were irradiated with a 200-watt Mazda lamp at a distance of 26 inches. When light was to be excluded the cultures were wrapped in black paper, only the cotton plug being left exposed, but in every other respect cultures grown in the light and in the dark were identical. *F. bulbigenum* var. *bataas* was grown on corn-meal agar, and one set of cultures was

<sup>4</sup> See footnote 2.



exposed to solar irradiation on the ledge of a north window. The dark cultures were wrapped in several thicknesses of black paper, except that the cotton plug was left free for the exchange of air.

Both organisms responded readily to solar irradiation. Pionnotes and sporodochia were produced more abundantly in the light than in the dark. When the organisms were grown in the dark, however, there was a larger production of mycelium.

Data were obtained from *Fusarium martii* var. *pisi* by combining the results from five and from six cultures grown on 2-percent potato-dextrose agar and cooked green bean pods, respectively, and from *Fusarium bulbigenum* var. *pisi* by combining the results from six cultures grown on corn-meal agar. Spore mounts were made as previously described, and the number of septations was determined for 1,000 and 1,200 spores produced by cultures on the various media grown in darkness and in light. The results are shown in table 8.

Table 8 shows that irradiation of the cultures of both species increased the number of septations. In all cases there was a much larger number of one- and two-septate conidia in the dark than in the light cultures. The highest percentage of four- and five-septate conidia occurred in cultures exposed to light, irrespective of the medium used.

TABLE 8.—Effect of light and darkness on conidial septation

Septation	Distribution of 1- to 5-septate conidia of—					
	<i>Fusarium martii</i> var. <i>pisi</i>				<i>Fusarium bulbigenum</i> var. <i>batatas</i>	
	Grown on cooked green bean pods under—		Grown on 2-percent potato-dextrose agar under—		Grown on corn-meal agar under—	
	Light	Darkness	Light	Darkness	Light	Darkness
1	26	221	14	114	0	925
2	24	138	3	142	0	64
3	1,035	839	895	744	407	187
4	115	2	88	0	576	24
5	0	0	0	0	217	0
Total.....	1,200	1,200	1,000	1,000	1,200	1,200

## DISCUSSION

Differences in results and conclusions due to individual workers cannot be eliminated entirely. With respect to the taxonomy of fungi and in particular of the genus *Fusarium* the personal factor is significantly important. Some of the results obtained by two investigators measuring conidia from the same microscopic mount were found to be significantly different, though not highly so, when treated statistically. If a significant difference, though small, occurs under conditions as uniform as those discussed above, it is not surprising that irreconcilable differences occur when the work is performed with little or no attention to uniformity of method.

A homogeneous dispersion of conidia of the two species studied was made from a single culture of the different media employed in which the proportion of three-, four-, and five-septate conidia in 10 different

mounts did not vary enough from the mean for the differences to be significant (tables 3 and 4).

It is evident that no general conclusions can be drawn for all species of *Fusarium* from the results obtained for one or two species alone. *Fusarium bulbigenum* var. *batatas* (table 5) was fairly consistent in its behavior when grown under uniform conditions, since there was no considerable deviation from the mean and none of the  $\chi^2$  values exceeded the 5-percent point. *F. martii* var. *pisi*, however, yielded large differences between cultures that appeared to be very uniform in growth, which suggests that enough cultures must be studied for the variations between tubes in respect to septations and spore sizes to be smoothed out.

The results indicate that light was an important factor in the production of what is known as "high culture" and in increasing the size and number of septations of the conidia. A small number of septations (1 and 2) predominated in cultures grown in the dark, and a larger number (3, 4, and 5) predominated in those grown in the light. No five-septate and very few four-septate conidia were found in any of the cultures grown in the dark, whereas in cultures grown in the light they were plentiful, especially in the case of *Fusarium bulbigenum* var. *batatas*, which consistently produces conidia having several septations.

#### SUMMARY

That different investigators measuring conidia of the same species of *Fusarium* from the same microscopic preparation may secure significantly different results is shown when these results are treated statistically. Groups of 50 spore measurements from the same culture may vary considerably, but 150 to 200 measurements constitute a fairly safe sample of a population if the spore suspensions are carefully prepared and mounted.

There was no significant difference in the ratio of three, four, and five septations of the conidia in different microscope mounts made from the same spore suspension. Highly significant differences, however, sometimes occurred between culture tubes of the same organism when grown on the same medium. A single tube culture cannot be assumed to be representative of a number of tube cultures.

Light has an important influence on the production of conidial septation in certain species of *Fusarium*. A larger number of septations predominated in cultures exposed to the light than in those grown in the dark; on the other hand, a larger number of one- and two-septate conidia predominated in cultures grown in the dark than in those grown in the light. Four- and five-septate conidia were few or entirely lacking in cultures grown in the dark, but they were plentiful in irradiated cultures.



# PREDICTION OF CULL FOLLOWING FIRE IN APPALACHIAN OAKS<sup>1</sup>

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## INTRODUCTION

The two most important causes of butt defect in eastern hardwoods are rot that develops from fire wounds and rot that starts in the parent stumps of sprouts and spreads into the new sprouts (5).<sup>3</sup> Butt rot has long been recognized as one of the major harmful effects of fire in hardwoods, but only recently have attempts been made to discover means of estimating and predicting the extent of such losses other than by ocular estimate. Improved means of appraising fire damage should lead to better allocation of fire-protection funds and to more equitable settlement of damage claims. A valid method for predicting cull following fire should also help guide salvage policy and marking practice in both timber sales and stand improvement work.

Earlier investigations of decay following fire in Delta hardwoods (2) and Appalachian hardwoods (3) suggested possible correlation between butt cull and a number of measurable factors, including age of tree, number of years since the fire, diameter of the tree when wounded, and width of wound. The present study was designed to incorporate those factors most highly correlated with butt cull and most easily measurable in the field into a statistical mechanism by which the amount of cull that will develop in oaks wounded by fire in the Appalachian mountain region can be predicted. This work is one phase of an extensive investigation of fire effects that is being carried on at the Appalachian Forest Experiment Station. The characteristics of the forests in the region where this work is centered, with particular regard to the causes and amount of cull due to heart rot, have been described by Hepting and Hedgecock (3).

## METHODS

The field data were collected during 1937 and 1938 on 11 commercial logging operations distributed as shown in figure 1. The number of trees examined on any one operation depended upon the size and speed of the logging job, the number of species on the area, and range in wound sizes and ages available. The crew followed as closely as possible behind the cutters, examining each stump for basal wounds, both healed and open. Where there was evidence of basal wounding,

<sup>1</sup> Received for publication October 1, 1940. The field work on which this paper is based was financed largely by the Appalachian Forest Experiment Station, Forest Service, U. S. Department of Agriculture. The data were collected by P. W. Warlick and K. H. Garren, temporary assistants, and E. R. Roth, assistant pathologist, attached to the Civilian Conservation Corps.

<sup>2</sup> Special acknowledgment is due Prof. F. X. Schumacher, of Duke University, for direction in the analysis of the data, and C. A. Abell, of the California Forest Experiment Station, formerly of the Appalachian station, and G. M. Jemison, of the Appalachian station, for their advice and cooperation.

<sup>3</sup> Italic numbers in parentheses refer to Literature Cited, p. 119.

a record was made of original width of wound at stump height, stump diameter at time of wounding, wound age (years since the fire), tree age, diameter at breast height, and volume of cull that resulted from the wound in board and cubic feet. Where the decay following wounding resulted in the complete loss of a section 6 feet or more in length, the cull section was scaled as a separate log. The volume of cull sections less than 6 feet in length was computed as a proportion of the volume of a 16-foot butt log. Logs were scaled by the International Log Rule. Rot not extensive enough to result in long butts was scaled out according to standard United States Forest Service instructions (7).

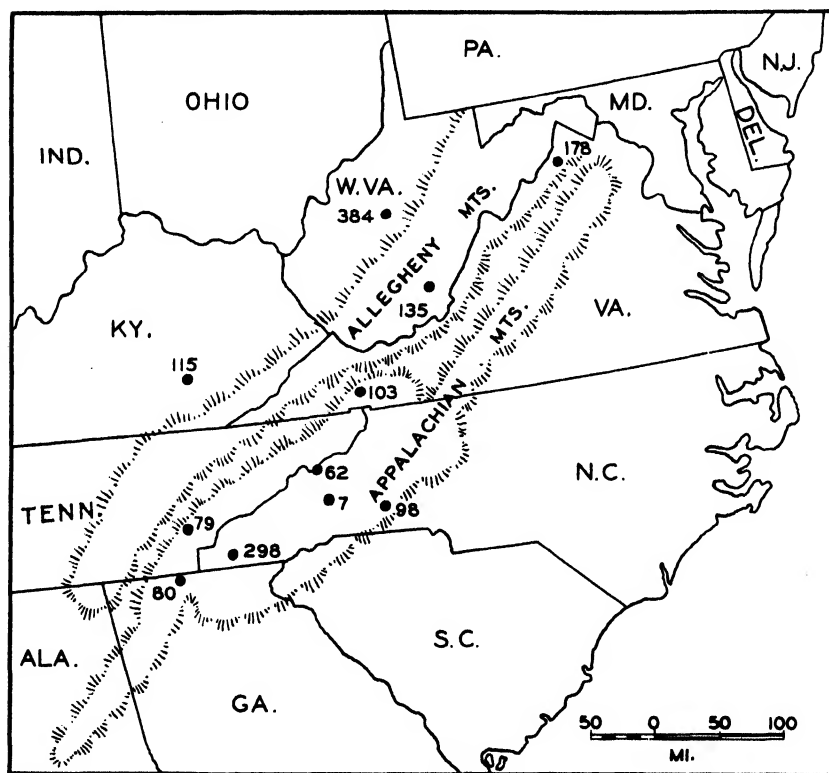


FIGURE 1.—Location of study areas and the number of trees analyzed in each area.

Occasionally rot was evident in a stump that had no indication of basal wounding. Such decay could usually be traced to a low healed wound that did not reach stump height, although in some cases the rot developed in the roots and worked up, and in some sprout trees the decay undoubtedly originated in the parent stumps. There were also cases in which severe decay or hollowing made it impossible to determine the width or age of the wound that led to the decay and sometimes obliterated the wound entirely. Trees upon which only fragmentary data or data requiring an undue amount of estimation

could be obtained, such as some of the types described above, were not recorded. The proportion of trees rejected was small, and the resulting bias was slight.

Although the original height of a basal wound affects the amount of cull that will develop, this factor could not be measured on most trees because it would have been necessary to split the butt logs and that could have been done only with cull sections. Wound width was therefore the only criterion of wound size obtained.

During 1937 the field work was confined to white oak on two stave operations. Since such trees were cut into short lengths, the amount of decay could be accurately measured. This work resulted in the standardization of methods, and in 1938 the work was extended to the other oaks, yellow poplar, and basswood on saw-timber operations. To date, sufficient data have been accumulated for the oaks only, and this paper will be confined to that group. The species studied were white oak (*Quercus alba* L.), chestnut oak (*Q. montana* Willd.), red oak (*Q. borealis maxima* (Marsh.) Ashe), black oak (*Q. velutina* LaM.), and scarlet oak (*Q. coccinea* Muench.).

#### DEVELOPMENT OF THE EQUATION

Preliminary analyses had established that the two measurable factors most highly correlated with butt-cull volume were wound width and wound age. In order to explore the interrelationships among these three variables, the data were grouped into broad classes of wound width and age and plotted as shown in figure 2. This

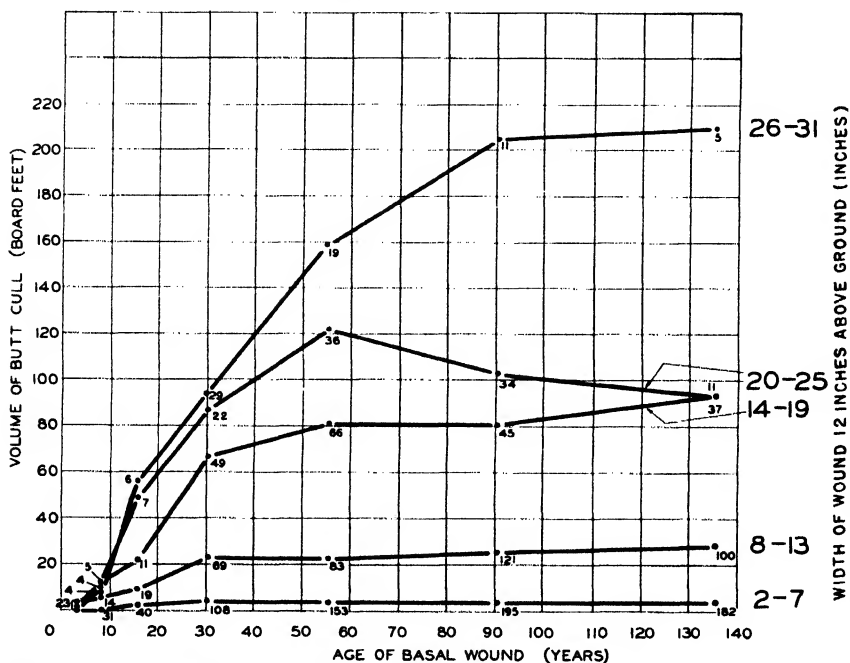


FIGURE 2.—Volume of butt cull in board feet plotted over age of basal wound for different wound widths. This figure is based on the raw data, combining all species of oak. The numbers beside the points indicate the number of trees on which each point is based.

figure is based upon the data from 892 white oaks, 297 chestnut oaks, 157 red oaks, 146 black oaks, and 47 scarlet oaks. It clearly indicates that under average conditions butt rot does not continue to extend indefinitely, but ceases after a certain number of years, depending in part upon the wound width. To provide a means of estimating cull for different wound widths and numbers of years following burning, it seemed desirable to express the relation of butt cull to wound width and wound age in the form of an equation. Since figure 2 indicates little increase in cull beyond 60 years except in the largest wounds, the equation was fitted only to the data through 60 years. It was thus possible to express the relation of cull to wound age in the form of a parabola. A curve expressing the relation of cull to wound width for the data up to 60 years indicated that cull increased with wound width at an increasing rate for narrow wounds, the rate becoming more or less constant for wounds of medium width and decreasing for wide wounds.

It thus appeared that the relation of cull to wound age could be expressed by an equation containing the first and second powers of age as follows:

$$Y = a + b_1A + b_2A^2 \quad (1)$$

where

$Y$  = cull in board feet

$a$  = a constant

$b_1, b_2$  = regression coefficients

$A$  = wound age

Analysis of very young wounds showed that at least 3 years must elapse before there is measurable cull due to rot behind fire wounds. Therefore, the condition is imposed upon equation (1) that when  $A = 3$ ,  $Y = 0$ , as follows:

$$\begin{aligned} 0 &= a + 3b_1 + 9b_2 \\ a &= -3b_1 - 9b_2 \end{aligned} \quad (2)$$

Now substituting the value for  $a$  in equation (2) for  $a$  in equation (1):

$$\begin{aligned} Y &= -3b_1 - 9b_2 + b_1A + b_2A^2 \\ Y &= b_1(A - 3) + b_2(A^2 - 9) \end{aligned} \quad (3)$$

As already mentioned, the relation of  $Y$  to  $A$  depends upon wound width ( $W$ ), and because of the shape of the wound width-cull curve it will take three powers of  $W$  to express this relation. Thus  $b_1$  and  $b_2$ , the terms denoting the slope of the wound age-cull curve, may be expressed as follows:

$$\begin{aligned} b_1 &= m + c_1W + c_2W^2 + c_3W^3 \\ b_2 &= n + d_1W + d_2W^2 + d_3W^3 \end{aligned} \quad (4)$$

where  $m$  and  $n$  are constants, and  $c_1, c_2, c_3, d_1, d_2$ , and  $d_3$  are regression coefficients.

Analysis of more than 1,000 wounds less than 4 inches in width showed that practically no cull resulted from wounds 3 inches or less in width measured about 12 inches above ground. Thus the condition is imposed upon equations (4) that when  $W = 3$ ,  $b_1$  and  $b_2 = 0$ :

$$\begin{aligned} 0 &= m + 3c_1 + 9c_2 + 27c_3 \\ m &= -3c_1 - 9c_2 - 27c_3 \\ \text{similarly } n &= -3d_1 - 9d_2 - 27d_3 \end{aligned} \quad (5)$$

Now substituting equations (5) for  $m$  and  $n$ , respectively, in equations (4):

$$\begin{aligned} b_1 &= -3c_1 - 9c_2 - 27c_3 + c_1W + c_2W^2 + c_3W^3 \\ b_1 &= c_1(W-3) + c_2(W^2-9) + c_3(W^3-27) \} \\ \text{similarly } b_2 &= d_1(W-3) + d_2(W^2-9) + d_3(W^3-27) \} \end{aligned} \quad (6)$$

Now substituting equations (6) for  $b_1$  and  $b_2$ , respectively, in equation (3) the final equation is obtained:

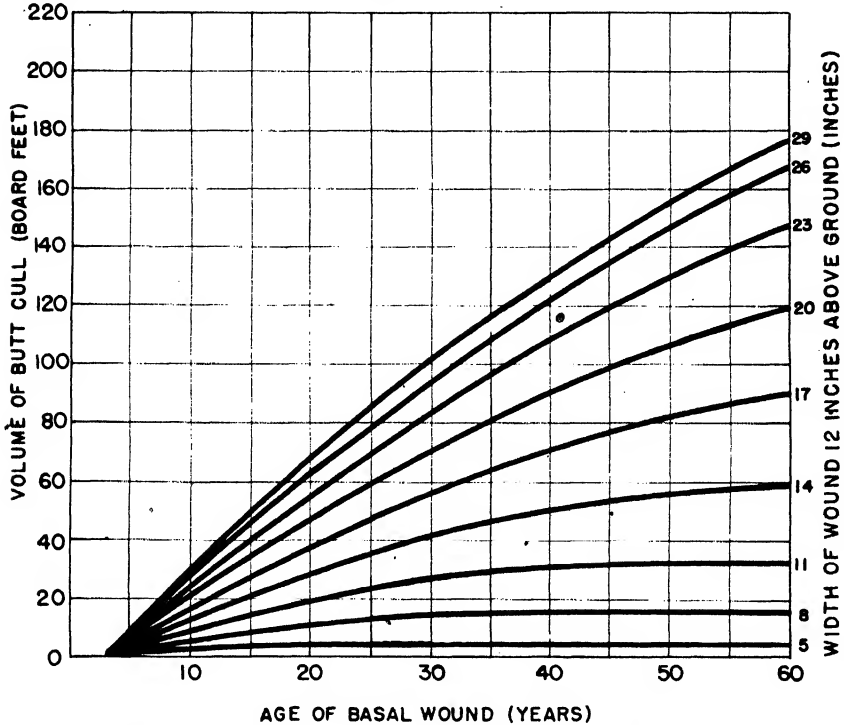


FIGURE 3.—Relation of butt-cull volume in board feet to age of basal wound for different wound widths. These curves were derived by multiple-regression analysis.

$$Y = c_1(W-3)(A-3) + c_2(W^2-9)(A-3) + c_3(W^3-27)(A-3) + d_1(W-3)(A^2-9) + d_2(W^2-9)(A^2-9) + d_3(W^3-27)(A^2-9).$$

The solution of this equation by the method of least squares gave the following for wound ages up to 60 years and widths up to 31 inches:

$$\begin{aligned} Y &= 0.1467 \pm 0.02876 (W-3) (A-3) \\ &+ .0040456 \pm .0020171 (W^2-9) (A-3) \\ &- .000115276 \pm .000052085 (W^3-27) (A-3) \\ &- .0043127 \pm .0005517 (W-3) (A^2-9) \\ &+ .000223 \pm .0000415 (W^2-9) (A^2-9) \\ &+ .0000038365 \pm .0000010952 (W^3-27) (A^2-9) \end{aligned}$$

The equation is graphically illustrated in figure 3. In the development of the equation, instead of computing squares and cross prod-



ucts for each tree, the data were grouped into nine classes of wound width and seven classes of age. The equation was derived from the class means, together with their appropriate frequencies. The standard error, computed directly from the residuals for each tree, is 48, which is 112 percent of mean  $Y$ . The equation accounts for 61 percent of the sum of squares of cull around zero. The magnitude of the standard error varies with estimated  $Y$ , as shown in figure 4. This regression (fig. 4) was determined by grouping the trees into classes of estimated  $Y$  and computing the standard deviation of residuals for each class.

In figure 5 the residuals are plotted about the net regression curves of cull over wound width and cull over wound age. These curves show that the equation type adopted is suitable and that the equation

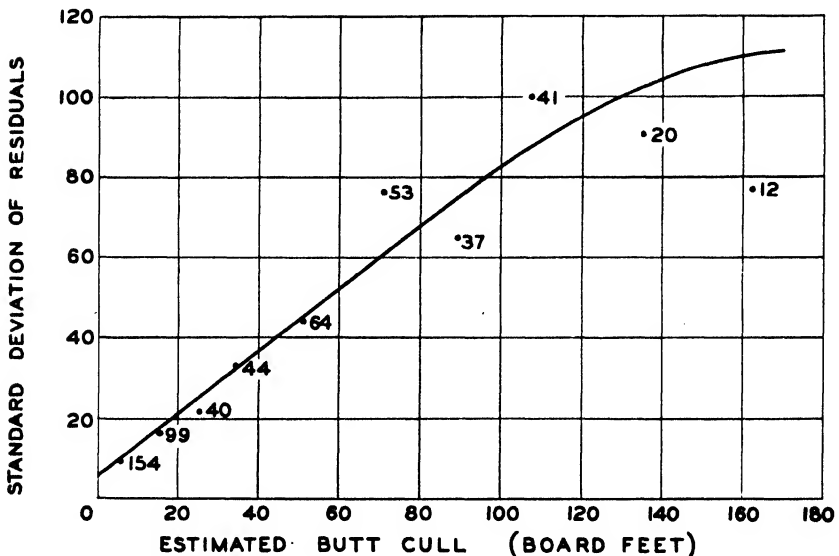


FIGURE 4.—Variation of the standard deviation of residuals with volume of butt cull as estimated from the regression equation. The numbers beside the points indicate the number of trees on which each point is based.

satisfies the data reasonably well. The unaccountably large amount of cull in the trees with wounds in the 40-year age class raised the general level of the curve in figure 5, *B*, above that defined by the other age classes.

The estimates of cull from figure 3 are not in close agreement with those of Hepting and Hedgecock (3, *fig. 6*). While in the earlier work wound age as a variable was not included and the graphs are thus not readily comparable with figure 3 of the present work, nevertheless estimates of cull from the earlier curves would run consistently higher than those from figure 3. The present curves, while yet perhaps not the final answer, should be regarded as superseding those given by Hepting and Hedgecock. Since the earlier work was not designed primarily to study decay behind basal wounds, it is possible that the smaller closed wounds on many of the sound trees were not recorded.

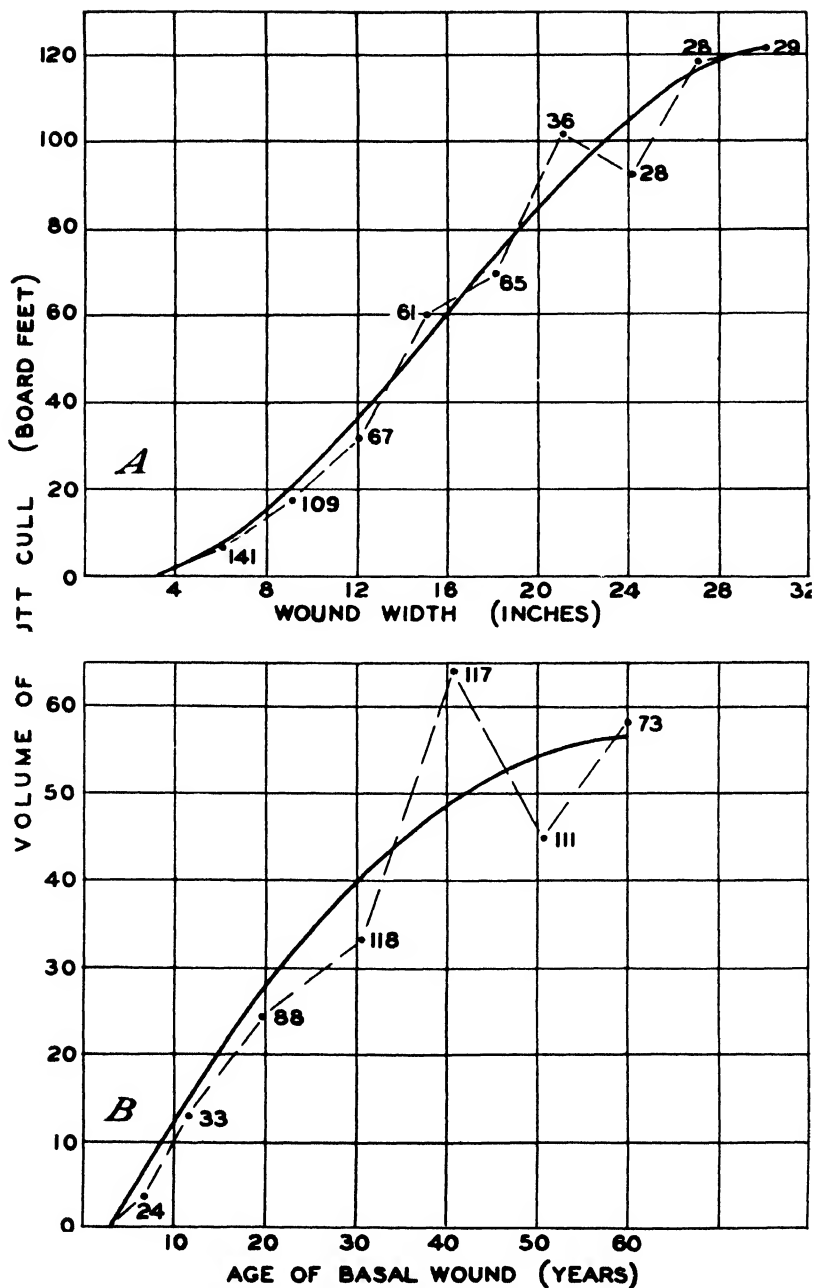


FIGURE 5.—Residuals plotted about the net regression of butt-cull volume over (A) wound width, for mean wound age, and (B) wound age, for mean wound width. The numbers beside the points indicate the number of trees on which each point is based.

## SIGNIFICANCE OF VARIABILITY IN CULL AMONG SPECIES, SITES, AND LOCALITIES

The possibility that mathematically significant differences in rate of decay existed among the five species of oak studied was tested by the analysis of variance of residuals after reducing them to standard units. The residual for each tree was divided by the standard deviation of residuals appropriate to its estimated cull, from figure 4, thereby putting the residuals in terms of standard units. Thus, the variances of residuals at different points along the regression lines covered essentially the same range and could be pooled into a general analysis.

Differences among species were tested separately for wounds up to 12 inches wide and for those 13 inches wide and over. In table 1 the residuals, by species and by two classes of wound width, are given. In neither case was the mean square among species significant at the 5-percent level by Snedecor's *F* test (6). Table 2 presents the analysis of variance for all wound widths combined. The failure of the mean square among species to appear significant does not necessarily mean that these five species decay at the same rate, but it means that from these data it cannot be demonstrated that differences exist within a reasonable degree of expectancy, because the variation in rate of decay within a species was as great as among species.

TABLE 1.—Mean residuals for the 5 species of oak, by wound-width classes, in terms of standard units<sup>1</sup>

Species	Wounds 4 to 12 inches wide		Wounds 13 to 31 inches wide		Total wounds	
	Number	Mean residual (standard units)	Number	Mean residual (standard units)	Number	Mean residual (standard units)
White oak.....	106	-0.15	134	+0.18	240	+0.03
Chestnut oak.....	84	-.11	68	-.08	152	-.10
Red oak.....	52	+.02	25	-.14	77	-.04
Black oak.....	40	-.13	32	-.30	72	-.20
Scarlet oak.....	20	+.01	3	+.03	23	+.01

<sup>1</sup> A plus sign indicates a mean cull volume greater than, and a minus sign, less than, estimated cull based on all species.

TABLE 2.—Analysis of variance in cull among 5 species of oak

Variance	Degrees of freedom	Sum of squares	Mean square	Odds that the factor is significant
Among species.....	4	3.7495	0.9374	<20 : 1
Within species.....	559	561.3608	1.0042	
Total.....	563	565.1103		

The method used to test differences in decay rate among the species of oak was also used to test the effect of site. The following mean residuals, expressed in standard units, indicated that site might have a significant effect: Stream bottom<sup>1</sup> -0.17, lower slope -0.11, middle

<sup>1</sup> Stream bottom includes land within 10 feet elevation of running water; ridge includes land within 10 feet elevation of a ridge top. The slope between stream bottom and ridge is equally divided into lower, middle, and upper slope. This method of expressing site was suggested by L. I. Barrett, of the Appalachian Forest Experiment Station, Forest Service, U. S. Department of Agriculture.

slope  $-0.06$ , upper slope  $-0.03$ , and ridge  $+0.11$ . The analysis of variance presented in table 3, however, fails to show the differences in decay rates among sites as significant. There is ample evidence that susceptibility to wounding by fire varies considerably among the species of oak (4) and among sites, but it is not possible to demonstrate through this investigation that wounds of the same size on the different oaks studied or on different sites will result in different amounts of cull.

TABLE 3.—*Analysis of variance in cull among 5 site qualities*

Variance	Degrees of freedom	Sum of squares	Mean square	Odds that the factor is significant
Among sites	4	2.4556	0.6139	<20 : 1
Within sites	559	562.6023	1.0064	
Total	563	565.0579		

The significance of differences in cull among the 11 study areas was tested in a manner similar to the tests for species and site. The mean residuals for the different areas, expressed in standard units, ranged from  $-0.18$  to  $+0.43$ . The analysis of variance (table 4) fails to show the differences in cull among localities to be significant. Certain individual differences in cull between areas with the most and the least cull were significant, but in general the differences were so small that the mean square among localities failed to show significance.

TABLE 4.—*Analysis of variance in cull among 11 localities*

Variance	Degrees of freedom	Sum of squares	Mean square	Odds that the factor is significant
Among localities	10	17.0051	1.7005	<20 : 1
Within localities	553	548.1317	0.9912	
Total	563	565.1368		

### FUNGI ISOLATED FROM BUTT DECAY

Perhaps the most important single factor contributing to the great variation in butt-cull volume among trees of the same species and with wounds of approximately the same size and age is the fungus succession in the wood behind the wounds. During the first few years the fruiting bodies of numerous fungi that rot dead sapwood commonly appear on the dead bark over basal wounds. During the first summer after a fire *Schizophyllum commune* Fr., *Parus stipticus* Bull. ex Fr., *Daldinia concentrica* (Bolt ex Fr.) Ces. and De Not., *Nummularia* sp., and an occasional *Stereum* or *Polyporus* commonly appear; the second year *Polyporus versicolor* L. ex Fr., *P. pargamensis* Fr., *Stereum rameale* Schw. ex Burt, *S. lobatum* Fr., and *Lenzites betulina* L. ex Fr. are the most common that occur; and by the third year *P. gilvus* Schw. ex Fr.

usually makes its appearance. These fungi are generally followed by the heart rotters, which rarely signify their presence by producing sporophores. In some cases, especially behind small wounds, the sap rot fungi will progress a short distance into the heartwood and no further decay will follow.

Apparently chance has much to do with which heart rot fungi become established behind any given wound in oaks. During this study cultures were prepared from the decaying wood behind a number of wounds where specimens could be obtained that gave promise of yielding pure cultures.<sup>5</sup> Table 5 includes a list of the fungi isolated and the maximum and the average height above ground that the decays extended. Although table 5 is based upon isolations from wounds of different widths and ages, it strongly suggests that the extent of decay is partly dependent upon the fungus involved. This had previously been demonstrated for a somewhat different group of fungi in the case of southern bottom-land hardwoods (2).

TABLE 5. *Fungi isolated from butt decay*

Group <sup>1</sup>	Fungus	Isolations	Height of evident decay	
			Maximum	Average
		Number	Feet	Feet
1	<i>Hydnium erinaceus</i> Bull. ex Fr.	14	21.0	8.8±1.36
	<i>Stereum frustulosum</i> Pers. ex Fr.	22	18.6	8.7±.83
	<i>Poria cocos</i> (Schw.) Wolf	4	8.3	7.4±.40
	<i>Polyporus berkeleyi</i> Fr.	8	8.8	6.7±.63
	<i>Polyporus frondosus</i> Dicks. ex Fr.	4	7.7	6.3±.69
	<i>Armillaria mellea</i> Vahl ex Fr.	4	5.3	4.2±.48
	<i>Poria andersonii</i> (Ell. and Ev.) Neuman	2	25.2	16.2
	<i>Polyporus spraguei</i> Burk. and Curt.	1	16.0	16.0
	<i>Polyporus dryophilus</i> Berk.	2	28.7	14.6
	<i>Polyporus dryadaceus</i> Pers. ex Fr.	2	15.9	10.5
	<i>Corticium leiodum</i> Pers. ex Fr.	1	10.5	10.5
	<i>Polyporus zonalis</i> Berk.	2	13.2	10.3
2	<i>Poria nigra</i> Berk.	3	10.0	8.7
	<i>Polyporus sulphureus</i> Bull. ex Fr.	2	7.5	6.0
	<i>Polyporus pseudo-sulphureus</i> Long	1	5.3	5.3
	<i>Flatulina hepatica</i> Huds. ex Fr.	1	3.7	3.7

<sup>1</sup> Group 1 includes only those fungi that are represented by 4 or more isolates, and for them the standard error of the average decay height is given; group 2 includes those fungi represented by less than 4 isolates, and for them no standard errors were computed.

### PREDICTION OF CULL

By the end of one growing season after a fire the limits of the basal wounds formed can be fairly accurately determined because of the raised callus ridges at the edges of the wounds and the dead, loosened bark. A tally of the new basal wounds according to their widths 12 inches above ground and the application of the equation illustrated in figure 3 afford a means of predicting the volume of cull that will follow. If the number of trees wounded and the wound widths are known, together with the approximate date of harvest, the expected board-foot loss at the time of harvest can be computed. If this volume is converted to dollars per acre, and the latter value discounted to the time of the fire at an appropriate interest rate, an estimate of the financial loss due to cull can be obtained. The greater the number of trees wounded, the greater the accuracy of prediction.

<sup>5</sup> Cultures were prepared from the specimens and determinations were made by R. W. Davidson, of the Division of Forest Pathology.

For example, cull predictions for a group of 500 trees would have, under average conditions, a standard error of about 5 percent, and for 1,000 trees, 3 percent.

Basal wounds should be tallied only on those trees that are already crop trees or that show fair prospect of becoming such. Hence no cull would be computed for the overtopped and part of the intermediate trees or trees already culled when wounded. Neither should wounds be tallied on trees already known to be butt-rotted. The examination of all crop trees on a burned area would ordinarily be unnecessary. Except in the case of very small fires, a 5- or 10-percent tally, by strips or plots, would probably suffice to sample the extent of wounding.

The integration of the fire losses due to cull, mortality, and other factors, leading to a complete analysis of damage from individual fires expressed in dollars per acre, is the goal of the series of investigations of which this is a part. A recent article by Bentley (1) illustrates the need for sound standardized methods of predicting fire damage in hardwoods, so that personal judgment will be reduced to a minimum. He cites a specific case in a cut-over hardwood stand in Tennessee, where the damage resulting from certain fires was greatly underestimated. His own method of damage appraisal, however, appears to lack factual basis and to provide estimates much too high.

#### SUMMARY

Investigation of decay that developed from basal fire wounds on oaks led to the development of a statistical mechanism by which the cull that follows a particular fire can be predicted. The predicting equation is for single trees and is based upon the width of basal wounds 12 inches above ground and the number of years since the fire. The data were collected on trees cut for lumber, cross ties, and staves at 11 localities in the Appalachian Mountains.

It could not be demonstrated that basal wounds of equal width produced significantly different amounts of cull among the species of oak, among the different sites, or among the study areas.

Much of the variation in amount of cull among trees with wounds of similar sizes and ages was due to the different fungi that became established. Of the 16 species of decay fungi isolated from decay following wounding, the most common were *Stereum frustulosum* and *Hydnum erinaceus*.

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# HYBRIDIZATION BETWEEN *USTILAGO AVENAE* AND *U. PERENNANS*<sup>1</sup>

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## INTRODUCTION

Numerous cases of hybridization between species (1, 4, 5, 6, 7)<sup>2</sup> and between genera (8) of the smut fungi have been reported in recent years. It is notable, however, that in all such instances the hybrids were produced by inoculating a host known to be common to the organisms involved in the cross. Thus far there has been no report of the production of hybrid chlamydospores in the smut fungi in which the species or genera involved in the cross did not have a common host. Recently, the writers have obtained results that seem to indicate definitely that hybrid chlamydospores were produced by crossing *Ustilago avenae* (Pers.) Jens. (loose smut of oats) and *U. perennans* Rostr. (loose smut of tall oatgrass (*Arrhenatherum elatius* (L.) Mert. and Koch)), two species for which no common host is known. These results are presented herein.

## MATERIAL AND METHODS

All cultures used in these investigations were of monosporidial origin and were obtained by isolating single sporidia from individual, germinating chlamydospores. Each chlamydospore from which sporidia were isolated was given a number and the four sporidia were numbered according to their respective positions on the promycelium, beginning at the distal end from the spore. For example, *Ustilago avenae* 52-1, 52-2, 52-3, 52-4 are the designations for monosporidial lines obtained from chlamydospore 52 of that species. The sex reaction of the various groups of monosporidial lines was determined by mating them in all possible combinations of two on plain agar and examining for sporidial fusions after 24 hours or longer. Only those combinations showing sporidial fusions in culture were used for inoculation purposes. In some of the tests the inoculum was composed of only two monosporidial lines, in which case the combination is indicated as 1×2 or 3×4, as the case may be, while in other instances the inoculum was composed of four lines, two of one sex and two of the opposite sex, in which case the combination is indicated as 1+2×3+4. In the case of an interspecies combination the culture number or numbers shown first represent the species listed first. For example, *U. avenae* 54×*U. perennans* 8 sporidial combination 1×3 denotes that culture No. 1 of *U. avenae* 54 was combined with culture No. 3 of *U. perennans* 8.

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<sup>2</sup> Italic numbers in parentheses refer to Literature Cited, p. 128.



The culture inoculum was prepared by mixing sporidia of opposite sex on 2-percent potato-dextrose agar and incubating at room temperature for 12 to 18 hours, at which time a sporidial suspension of the combined cultures was made in sterile tap water. Chlamydospore inoculum consisted of a suspension of spores in tap water made immediately before inoculation. Inoculations were made by the partial-vacuum method described by Allison (1). The oat seed was soaked in formaldehyde solution (1:320) for 10 minutes to kill any smut that might be present as a result of field inoculation and it was then washed with running water to remove the formaldehyde. The tall oatgrass seed was given the same formaldehyde treatment for 1 hour.

## RESULTS

### PATHOGENICITY OF INTRASPECIES AND INTERSPECIES HYBRIDS

Seed of Victory oat (C. I.<sup>3</sup> 560) (*Avena sativa* L.) and wild oats (*A. fatua* L.) was inoculated with intraspecies and interspecies sporidial combinations of *Ustilago avenae* and *U. perennans* and planted in the greenhouse in the winter of 1937-38. Both hosts were infected by *U. avenae*, neither host was infected by *U. perennans*, and slight infection was obtained on wild oats only with the interspecies combinations. The interspecies F<sub>1</sub> chlamydospores were echinulate, resembling both parents in this respect. Furthermore, they were capable of normal germination, but the sporidia, with few exceptions, were incapable of growth on culture media.

In view of these preliminary results, the inoculations were repeated and the seed was planted in the field in the spring of 1938. The results, presented in table 1, substantiate those obtained from the preliminary inoculations. *Ustilago avenae* produced 60 and 90 percent smut on Victory and wild oats, respectively, while *U. perennans* produced no smut on either of these hosts. The two interspecies crosses failed to infect Victory oats but produced slight infection (0.8 and 3.2 percent) on wild oats. As shown in table 1, no results were obtained from the inoculations of tall oatgrass because the plants did not head. Again it was found that the hybrid chlamydospores produced on wild oats germinated normally, but the sporidia would not grow in culture.

The F<sub>1</sub> chlamydospores of the interspecies crosses were used to inoculate seed of the three hosts which was planted in the greenhouse in the fall of 1938. The results of these inoculations are also presented in table 1. Both Victory oats and wild oats were infected by the two hybrids, one hybrid producing 96 and 92 percent smut and the other 38.5 and 16.5 percent smut on these two hosts, respectively. In contrast, tall oatgrass was not infected by either hybrid. The failure to recover a *Ustilago perennans* segregate on tall oatgrass is difficult to explain, especially in view of the fact that a *U. avenae* segregate apparently was recovered on Victory oats. Furthermore, the evidence that *U. perennans* actually was one parent of the two hybrids is entirely of the negative type, being based on the fact that Victory oats and wild oats, both of which are highly susceptible to *U. avenae*, were not infected by the *U. perennans* inoculum. However, a new series of inoculations on seed planted in the greenhouse in the fall of 1938 gave results that substantiate those described above.

<sup>3</sup> C. I. refers to accession number of the Division of Cereal Crops and Diseases.

TABLE 1.—Smut resulting from inoculations with intraspecies and interspecies sporidial combinations and  $F_1$  hybrid chlamydospores of *Ustilago avenae*  $\times$  *U. perennans*

Species and cross	Sporidial combination	Inoculum	Smuted panicles in—		
			Victory oats	Wild oats	Tall oatgrass
			Percent	Percent	Percent
<i>U. avenae</i> 52	1+4 $\times$ 2+3	Sporidia	60.0	90.0	(1)
<i>U. perennans</i> 5	1+3 $\times$ 2+4	do.	0.0	0.0	(1)
	1+4 $\times$ 2+4	do.	0.0	0.8	(1)
	2+3 $\times$ 1+3	do.	0.0	3.2	(1)
<i>U. avenae</i> 52 $\times$ <i>U. perennans</i> 5	1+4 $\times$ 2+4	$F_1$ chlamydospores	96.0	92.0	0.0
	2+3 $\times$ 1+3	do.	38.5	16.5	0.0

<sup>1</sup> Inoculations were made, but the plants failed to head.

In the second series of inoculations, cultures of two races of *Ustilago avenae* and of one race of *U. perennans* were used and the inoculum in each case was composed of compatible combinations of two monosporidial lines, both within a species and between species. Inoculations were made on Anthony oats, wild oats, and tall oatgrass, except that tall oatgrass was not inoculated with *U. avenae*, and Anthony oats were not inoculated with *U. perennans* and the interspecies combinations. The results of these inoculations are presented in table 2. The efficiency of the inoculation process and the fact that optimum conditions for infection prevailed are well established by the results obtained with Anthony oats. Six of the eight sporidial combinations of *U. avenae* used to inoculate this variety produced 100 percent infection and the other two produced more than 90 percent infection. The susceptibility of wild oats to the *U. avenae* inoculum was shown by the fact that one of the races produced 83 percent and the other race 82 percent infection on this host. The inoculation of tall oatgrass and wild oats with *U. perennans* resulted in infection of the former by all four sporidial combinations used, the estimated percentages ranging from 15 to 35, while no smut was produced on wild oats by any of the inoculations with this species.

In table 2 it can be seen that of the 16 interspecies combinations used to inoculate wild oats and tall oatgrass, 4 produced smut on the former while none produced smut on the latter. Sporidium No. 1 of *Ustilago avenae* 54 crossed with sporidium No. 3 of *U. perennans* 8 produced 83.3 percent smut on wild oats and sporidium No. 4 crossed with sporidium No. 3, of the same two species, respectively, produced 53.8 percent smut on wild oats. Likewise the same sporidial combinations, i. e., 1  $\times$  3 and 4  $\times$  3, of *U. avenae* 56 and *U. perennans* 8, produced 10 percent and 18.1 percent smut, respectively, on wild oats. In this connection it should be noted, as shown in table 2, that the 4 interspecies combinations in which the sporidia fused and subsequently produced infection hyphae in culture produced smut on wild oats, while no infection resulted from the inoculations with those sporidial combinations in which the sporidia fused without the subsequent production of infection hyphae. Thus, apparently there were two degrees of compatibility in the interspecies combinations of opposite sex, represented on the one hand by sporidial fusions

without further development in culture, and on the other hand by sporidial fusions followed by the production of infection hyphae and ultimately the development of chlamydospores on the host.

TABLE 2.—*Smut resulting from inoculations with intraspecies and interspecies crosses of Ustilago avenae and U. perennans*

Species and cross	Sporidial combination	Sex reaction in culture <sup>1</sup>	Smutted plants <sup>2</sup> in —		
			Anthony oats	Wild oats	Tall out-grass
			Percent	Percent	Percent
<i>U. avenae</i> 54	1 × 2	++	100.0	83.0	
	1 × 3	++	100.0		
	2 × 4	++	100.0		
	3 × 4	++	91.0		
<i>U. avenae</i> 56	1 × 2	++	95.0	82.0	
	1 × 3	++	100.0		
	2 × 4	++	100.0		
	3 × 4	++	100.0		
<i>U. perennans</i> 8	1 × 3	++		0.0	30.0
	1 × 4	++		0.0	15.0
	2 × 3	++		0.0	35.0
	2 × 4	++		0.0	30.0
<i>U. avenae</i> 54 × <i>U. perennans</i> 8	1 × 3	++		83.3	0.0
	1 × 4	+		0.0	0.0
	2 × 1	+		0.0	0.0
	2 × 2	+		0.0	0.0
	3 × 1	+		0.0	0.0
	3 × 2	+		0.0	0.0
	4 × 3	++		53.8	0.0
	4 × 4	+		0.0	0.0
<i>U. avenae</i> 56 × <i>U. perennans</i> 8	1 × 3	++		10.0	0.0
	1 × 4	+		0.0	0.0
	2 × 1	+		0.0	0.0
	2 × 2	+		0.0	0.0
	3 × 1	+		0.0	0.0
	3 × 2	+		0.0	0.0
	4 × 3	++		18.1	0.0
	4 × 4	+		0.0	0.0

<sup>1</sup> The double plus sign (++) indicates sporidial fusions with the subsequent production of infection hyphae, while the single plus sign (+) indicates sporidial fusions without the subsequent production of infection hyphae.

<sup>2</sup> Based on plant counts, the total number ranging from 15 to 24 in Anthony oats and 9 to 15 in wild oats, except in tall outgrass, where the percentages were estimated.

In view of the apparent difference in degree of compatibility of the interspecies sporidial combinations, another inoculation test was made in which only those combinations were used for inoculum in which fused sporidia produced infection hyphae, and only one host, wild oats, was inoculated. In addition, the F<sub>1</sub> chlamydospores of the interspecies hybrids shown in table 2 were used to inoculate Anthony oats and wild oats, which were seeded in the field in the spring of 1939. The results are presented in table 3. The susceptibility of the wild oats to the *Ustilago avenae* inoculum is shown by the production of 85, 80, 50, and 77.6 percent smut by the four combinations of monosporidial lines used. On the other hand, *U. perennans* produced no smut on this host. All of the sporidial combinations involving *U. avenae* 54, 56, and 57 with *U. perennans* 8 produced smut, the percentages ranging from 2.5 to 90. Of the four sporidial combinations between *U. avenae* 55 and *U. perennans* 8, only one, 4 × 3, produced smut (47.2 percent). There is no apparent explanation for the failure of the other three combinations to produce smut, as infection hyphae were produced by the fused sporidia on plain agar. Likewise it is difficult to explain the wide differences in the amounts of smut produced by other interspecies combinations shown in table 3. It is hoped that further studies will make possible an explanation of these results.

The results of the inoculations with  $F_1$  chlamydospores of the four interspecies hybrids show a high degree of infection (50 to 95.2 percent) with three of the hybrids, and low percentages of infection (7.5 to 7.9 percent) with the other hybrid (table 3). Thus, segregates having a pathogenicity similar to that of the *Ustilago avenae* parent were recovered on Anthony oats in the  $F_2$  generation, although no smut was produced on this variety in the  $F_1$  generation (table 2). The results of inoculations on tall oatgrass with these four hybrids are under investigation.

TABLE 3.—Smut resulting from inoculations with intraspecies and interspecies sporidial combinations and  $F_1$  hybrid chlamydospores of *Ustilago avenae* and *U. perennans*

Species and cross	Sporidial combination	Sex reaction in culture	Inoculum	Smutted panicles <sup>1</sup> in—	
				Anthony oats	Wild oats
				Percent	Percent
<i>U. avenae</i> 54	1+4×2+3	++	Sporidia		85.0
<i>U. avenae</i> 56	1+4×2+3	++	do		80.0
<i>U. avenae</i> 55	1+2×3+4	++	do		50.0
<i>U. avenae</i> 57	1+3×2+4	++	do		77.6
<i>U. perennans</i> 8	1+2×3+4	++	do		0.0
<i>U. avenae</i> 54× <i>U. perennans</i> 8	1×3	++	do		46.7
	4×3	++	do		86.0
<i>U. avenae</i> 56× <i>U. perennans</i> 8	1×3	++	do		83.3
	4×3	++	do		16.4
	3×1	++	do		4.2
<i>U. avenae</i> 57× <i>U. perennans</i> 8	3×2	++	do		2.5
	4×3	++	do		90.0
	4×4	++	do		3.3
	1×1	++	do		0.0
<i>U. avenae</i> 55× <i>U. perennans</i> 8	1×2	++	do		0.0
	4×3	++	do		47.2
	4×4	++	do		0.0
<i>U. avenae</i> 54× <i>U. perennans</i> 8	1×3		$F_1$ chlamydospores	62.1	92.3
	4×3		do	50.0	95.2
<i>U. avenae</i> 56× <i>U. perennans</i> 8	1×3		do	7.9	7.5
	4×3		do	61.1	78.1

<sup>1</sup> Based on total panicles in each 5-foot row in the field.

#### COMPATIBILITY OF MONOSPORIDIAL COMBINATIONS

The difference in degree of compatibility between the combinations of monosporidial cultures previously described is substantiated by results obtained with combinations of two other monosporidial complements of *Ustilago perennans* with *U. avenae*, *U. levis* (Kell. and Sw.) Magn., a buff strain of *U. levis*, and *U. hordei* (Pers.) Kell. and Sw.

*Ustilago perennans* 5 and 6, each represented by full complements of four pedigreed monosporidial cultures, exhibited nothing unusual when mated with each other in all possible combinations, as shown in table 4. The eight monosporidial lines all seemed equally able to fuse with others of opposite sex, and fusion was followed by the normal development of infection hyphae. However, when these same eight cultures were paired with cultures of the other species, a compatibility difference, which is linked with the sex involved, became evident. The results of these matings are recorded in table 5.

In each of the two complements of pedigreed monosporidial cultures of *Ustilago perennans* there are two cultures that are more active in interspecies matings than the other two, and, also, in each case the two stronger cultures are of the same sex. In *U. perennans* 5, it is

cultures 1 and 2 in each instance that participate in the development of infection hyphae with whatever sporidia of opposite sex they are paired. Sporidia of the other two cultures, 3 and 4, fused with sporidia of opposite sex of the other species, but the union was seldom followed by the production of infection hyphae. In *U. perennans* 6, cultures 3 and 4 were the more active, and here again it is notable that these two are of the same sex. Furthermore, it is seen that in *U. perennans* 6, cultures 3 and 4 are of the same sex as *U. perennans* 5, cultures 1 and 2, as indicated by the results of cross-pairing these cultures.

TABLE 4.—Normal reaction obtained when two complements of pedigreed monosporidial cultures of *Ustilago perennans* are paired with each other on plain water agar

[++ = infection hyphae produced by fused sporidia; — = no reaction]

Chlamydospore No.	Sporidial No.	Sporidial No.							
		<i>U. perennans</i> 5				<i>U. perennans</i> 6			
		1	2	3	4	1	2	3	4
<i>U. perennans</i> 5.....	1	—	—	++	++	++	++	—	—
	2	—	—	++	++	++	++	—	—
	3	++	++	—	—	—	—	++	++
	4	++	++	—	—	—	—	++	++
<i>U. perennans</i> 6.....	1	++	++	—	—	—	—	++	++
	2	++	++	—	—	—	—	++	++
	3	—	—	++	++	++	++	—	—
	4	—	—	++	++	++	++	—	—

TABLE 5.—Anomalous sex reaction obtained when two complements of pedigreed monosporidial cultures of *Ustilago perennans* were paired with *U. avenae*, *U. levis*, and *U. hordei* on plain water agar

[++ = infection hyphae produced by fused sporidia; + = fusions, but no infection hyphae produced; — = no fusions]

Chlamydospore No.	Sporidial No.	Sporidial No.							
		<i>U. perennans</i> 5				<i>U. perennans</i> 6			
		1	2	3	4	1	2	3	4
<i>U. avenae</i> 33 .....	1	++	++	—	—	—	—	++	++
	2	—	—	+	+	+	+	—	—
	3	—	—	+	+	+	+	—	—
	4	++	++	—	—	—	—	++	++
<i>U. levis</i> 77 .....	2	++	++	—	—	—	—	++	++
	3	—	—	+	+	+	+	—	—
	4	—	—	+	+	+	+	—	—
	1	++	++	—	—	—	—	++	++
<i>U. levis</i> buff 47.....	2	++	++	—	—	—	—	++	++
	3	—	—	+	+	+	+	—	—
	4	—	—	+	+	+	+	—	—
	1	—	—	+	+	+	+	—	—
<i>U. levis</i> buff 50.....	2	++	++	—	—	—	—	++	++
	3	++	++	—	—	—	—	++	++
	4	—	—	+	+	+	+	—	—
	1	—	—	+	+	+	+	—	—
<i>U. hordei</i> 5.....	2	++	++	—	—	—	—	++	++
	3	—	—	+	+	+	+	—	—
	4	—	—	+	+	+	+	—	—

It seems apparent from the results given above that three types of reaction are manifest when monosporidial cultures of *Ustilago perennans* are mated with monosporidial lines of certain other *Ustilago* spp.: (1) Negative reaction, resulting from pairing two cultures of the same sex; (2) fusion between pairs of sporidia of opposite sex, and the subsequent development of infection hyphae; and (3) fusion of sporidia of opposite sex, but not followed by the production of infection hyphae. Although the last two types of reaction result from pairing cultures of opposite sex, apparently there also is involved some difference in the degree of compatibility between the two sexes. Similar differences were noted by Fischer (3) when monosporidial cultures of *U. striaeformis* (Westd.) Niessl. were paired with monosporidial cultures of *U. bullata* Berk. Bauch (2) reported much the same phenomenon in *Sphacelotheca schweinfurthiana* (v. Thüm.) Sacc., in which case intraspecies matings exhibited different types of reaction: in one type ("W-Reaktion") no further development of the fused sporidia took place, whereas in the other ("S-Reaktion") binucleate infection hyphae developed from the fused sporidia in the normal manner.

The basis for this anomalous sexual behavior is very difficult to determine. Some of the interspecies sporidial combinations were more compatible than others, and furthermore it was always the same two cultures in each set of four that were active enough to participate in the formation of infection hyphae. Perhaps it is significant that in each case the sporidia of the two more active cultures of a complement of four were of the same sex, not only with reference to their sister cultures but also with reference to the two more active cultures of another complement of four. The phenomenon is made still more complicated by the fact that in intraspecies matings between cultures of *Ustilago perennans* these compatibility differences have not been observed.

#### SUMMARY

*Ustilago avenae* and *U. perennans*, which do not have a known common host, were hybridized by inoculating wild oats with compatible monosporidial combinations of the two species. The wild oats were infected, but no infection was obtained from similar inoculations on Victory oats and tall oatgrass. Inoculations with *U. avenae* produced infection on Victory oats and wild oats but not on tall oatgrass, while *U. perennans* infected tall oatgrass but not Victory oats or wild oats.

Segregates having a pathogenicity similar to that of the *Ustilago avenae* parent were recovered from the hybrid chlamydospores by inoculating Anthony oats, but segregates behaving like the *U. perennans* parent were not recovered from similar inoculations on tall oatgrass.

When the sexually opposite monosporidial lines of *Ustilago perennans* were paired with *U. avenae*, *U. levis*, and *U. hordei*, two different degrees of compatibility were exhibited, viz, fusion of sporidia followed by the production of infection hyphae and fusion of sporidia without the subsequent production of infection hyphae. Infection of wild oats was produced by the former type, but not by the latter.

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## RESPONSE OF PHYMATOTRICHUM OMNIVORUM TO CERTAIN TRACE ELEMENTS<sup>1</sup>

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### INTRODUCTION

The cotton root rot disease, caused by *Phymatotrichum omnivorum* (Shear) Duggar, is one of the most serious plant diseases occurring in the southwestern part of the United States. Investigations by numerous workers over the last half century suggest that this disease-producing fungus is not amenable to the methods of control commonly and successfully applied to other plant pathogens. It is evident that a more exact knowledge of the relation of the organism to its environment, both biological and chemical, must be obtained before a satisfactory explanation can be had of the many obscure problems dealing with the organism and its action upon the host plant. The studies here reported constitute one phase of a general investigation of the nutritional requirements of *P. omnivorum* and deal primarily with the response of the organism to a number of the trace elements, including copper, iron, manganese, zinc, aluminum, boron, cadmium, cobalt, fluorine, mercury, iodine, lithium, molybdenum, nickel, and silicon. While the possibility that certain of these elements may be highly toxic to the root rot organism is a matter of practical interest, it is equally important to know the contribution of each of the elements to an optimum nutrient solution. Such information may be used in connection with the general problem of nutrition and other physiological investigations.

In the extensive literature dealing with cotton root rot and its causal organism, little is to be found regarding the effect of these elements on the fungus. Rogers (5)<sup>3</sup> in laboratory experiments found that copper had a stimulating effect at low concentrations but was toxic at higher concentrations, zinc stimulated growth at all concentrations up to and including 200 p. p. m., while aluminum and mercury were toxic at concentrations above 50 p. p. m. Taubenhaus, Ezekiel, and Fudge (10) reported that the addition of manganese,

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<sup>3</sup> Italic numbers in parentheses refer to Literature Cited, p. 159.



sulfur, iodine, iron, nickel, copper, and boron to soils was without effect on the incidence or severity of root rot.

The investigations of Steinberg (7, 8, 9) with *Aspergillus niger* Van Tiegh. have furnished considerable information on the influence of many of the elements upon that fungus. With the refinement by Steinberg of the technique for the removal of traces of impurities from the nutrient solution it has become possible to study more precisely the effect of the addition of the various elements upon fungus growth. Foster (4) has recently reviewed the literature dealing with the effects of heavy metals or trace elements on various fungi.

### MATERIALS AND METHODS

Preliminary experiments demonstrated that the nutrient solution designated as No. 70 by Ezekiel, Taubenhaus, and Fudge (2) was very favorable for growth of the root rot fungus, and with but slight modification it has been used in these studies, as follows:

Constituent:	Grams per liter
NH <sub>4</sub> NO <sub>3</sub> .....	1.00
K <sub>2</sub> HPO <sub>4</sub> .....	1.35
MgSO <sub>4</sub> .7H <sub>2</sub> O.....	.75
KCl.....	.15
FeCl <sub>3</sub> .6H <sub>2</sub> O.....	.00223
Glucose.....	40.0
Redistilled water to make to 1,000 cc.	

The following salts were used in studying the various trace elements: Copper sulfate (CuSO<sub>4</sub>), iron sulfate (FeSO<sub>4</sub>.7H<sub>2</sub>O), manganese sulfate (MnSO<sub>4</sub>.4H<sub>2</sub>O), zinc sulfate (ZnSO<sub>4</sub>.7H<sub>2</sub>O), aluminum sulfate (Al<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub>), potassium borate (K<sub>2</sub>B<sub>4</sub>O<sub>7</sub>.5H<sub>2</sub>O), cobalt sulfate (CoSO<sub>4</sub>.7H<sub>2</sub>O), potassium fluoride (KF.2H<sub>2</sub>O), mercuric chloride (HgCl<sub>2</sub>), potassium iodide (KI), lithium sulfate (Li<sub>2</sub>SO<sub>4</sub>.H<sub>2</sub>O), ammonium molybdate ((NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>.4H<sub>2</sub>O), nickel sulfate (NiSO<sub>4</sub>.6H<sub>2</sub>O), sodium silicate (Na<sub>2</sub>SiO<sub>3</sub>.9H<sub>2</sub>O), and cadmium sulfate (CdSO<sub>4</sub>).

In order to evaluate the effect of the addition of each of these elements to the nutrient solution, it was necessary to remove, so far as possible, all traces of these elements that might have been introduced into the solution as impurities in the c. p. grade of chemicals used in the preparation of the solution. For such purification, calcium carbonate was added to the solution at the rate of 15 gm. per liter, followed by autoclaving and filtering through fritted glass crucibles, after the method of Steinberg (7). To the filtered aliquots of the purified solution the elements were added, and the solution was then divided into 50-cc. portions in 250-cc. Erlenmeyer flasks and autoclaved at 15 pounds pressure for 15 minutes. The treatments were ordinarily set up in series of five flasks.

The flasks of nutrient solution were inoculated with 5-mm. disks of mycelium and adhering agar from potato-dextrose agar plate cultures of the organism. The dry weight of the inoculum piece was usually less than 5 mg., varying with the depth of the agar in the transfer plate. In the preliminary phases of the study a pure culture of *Phymatotrichum omnivorum* designated as isolate 41 was used, but in the later and more extensive experiments a newly isolated culture designated as isolate 28 was the source of the inoculum. Both cultures were of proved pathogenicity upon cotton seedlings.

The incubation of all cultures was at 28° C. in incubators or in a constant-temperature room, the period of incubation being 28 days in the majority of the experiments. The acidity of the solution in the culture flasks was determined with a Beckmann pH meter prior to inoculation and in many of the experiments at time of harvest. The fungus mats were harvested by filtering through Alundum crucibles and drying to constant weight at 80° C., either in crucibles or in weighing bottles. The dry weight of the fungus mat was the criterion of response to the addition of the various elements.

## RESULTS

### EFFECT OF ADDING ELEMENTS SINGLY

The effect of adding certain elements (copper, iron, manganese, and zinc) to the purified nutrient solution was studied in a series of experiments, the range of concentrations being from 0.5 to 160 p. p. m. In the upper portion of table 1 are presented the data from one typical experiment in the lower range of concentrations. With the addition of iron, manganese, or zinc, increases in mat weight were obtained over that of the control solution to which no element was added. The addition of copper at the rates of from 0.5 to 10 p. p. m. had no appreciable effect upon the mat weight; at a concentration of 20 p. p. m. growth was almost completely inhibited.

Other experiments were conducted in which the addition of each of these elements to the purified solution was studied at higher rates. The addition of copper at 40, 80, or 160 p. p. m. completely inhibited growth of the organism. Iron or manganese at these rates resulted in decreases in the amount of mat weight as the concentration increased, eventually yielding less than the control series. With the addition of zinc at similar rates, decreases resulted at the higher concentrations, but the weights were superior to those of the control flasks. It was noted that at concentrations beyond 20 p. p. m. a precipitate was formed in the nutrient-solution flasks following autoclaving, and it is probable that with this slightly acid nutrient solution only a part of the addition was held in solution. Therefore, with the heavy rates of addition the amount of the element remaining in solution is not known.

The determinations of pH values of the solutions prior to inoculation showed that the reaction of those solutions receiving zinc or manganese deviated only slightly from that of the solution (table 1) to which no metals were added. The addition of iron or copper resulted in a slightly more acid condition. The final pH values of each flask at time of harvest showed a general trend toward increased acidity. In this experiment as well as in others, no relationship was found between the initial pH value of the solution and the mat weight. The possibility of such a relation in solutions having a greater initial range of pH values is not excluded, but with the nutrient solution used and an initial pH value of from 5.5 to 6.5 following the addition of the several elements, the effect of slight variation in the initial pH value upon subsequent growth has been greatly overshadowed by the effect of the element or combination of elements employed in the solution. It has been reported by other workers with this organism and with practically the same nutrient solution (2) that *Phymatotrichum omnivorum* will grow in solutions of widely varying hydrogen-ion

concentration, and that, although the optimum pH value for growth appeared to be on the alkaline side of neutrality, growth was almost equally good over a range of approximately pH 4.0 to 9.0. In the present study no attempt was made to adjust the pH value of the nutrient solution.

TABLE 1.—Effect of adding certain elements, singly and at several rates, to a purified solution on the growth of *Phymatotrichum omnivorum* at 28° C. for 28 days

Element added	Rate of addition	Original pH	Final pH of replicate—				Mat weight				
							Replicate—				Mean
			a	b	c	d	a	b	c	d	
	<i>P.p.m.</i>						<i>Mg.</i>	<i>Mg.</i>	<i>Mg.</i>	<i>Mg.</i>	<i>Mg.</i>
Copper	0.5	5.92	5.70	5.83	5.60	5.79	166.7	139.8	159.7	169.6	159.0
	1.0	5.88	5.53	5.57	5.72	6.02	195.7	196.4	168.0	126.7	171.7
	2.0	5.83	5.65	5.71	5.50	5.96	167.3	197.2	90.5	113.1	142.0
	5.0	5.83	5.62	5.55	5.55	5.58	193.2	185.2	176.1	120.2	168.7
	10.0	5.80	5.61	5.37	5.40	5.45	179.0	149.4	158.3	141.9	157.2
Iron	20.0	5.65	4.93	4.45	4.42	4.42	36.2	41.1	40.2	45.0	40.6
	.5	6.04	5.60	5.79	5.84	5.67	274.0	229.7	272.8	241.7	254.6
	1.0	6.01	5.93	5.84	5.80	5.72	260.3	218.0	220.0	243.0	235.3
	2.0	5.99	5.34	5.62	5.66	5.57	304.9	229.9	327.4	202.7	266.2
	5.0	5.94	5.49	5.40	5.56	5.50	224.0	265.0	235.0	246.9	242.7
Manganese	10.0	5.93	5.40	5.34	5.19	5.23	245.4	220.7	150.6	189.6	201.6
	20.0	5.90	5.51	5.55	5.06	4.92	186.8	186.5	196.5	177.2	186.8
	.5	6.10	5.28	5.40	5.61	5.48	265.6	255.1	280.4	271.6	268.2
	1.0	6.13	5.30	5.47	5.50	5.54	259.0	246.8	247.3	240.4	248.4
	2.0	6.10	5.58	5.51	5.50	5.69	194.5	186.6	214.2	177.8	193.3
Zinc	5.0	6.17	5.52	5.50	5.39	5.51	160.4	232.1	170.9	120.6	172.5
	10.0	6.20	5.64	5.36	5.50	5.34	172.3	198.8	252.0	174.7	190.5
	20.0	6.13	5.20	5.27	5.44	5.33	252.4	231.9	230.4	251.6	241.6
	.5	6.14	5.60	6.21	6.48	4.26	345.1	315.1	371.3	406.9	359.6
	1.0	6.20	4.49	5.01	4.92	4.65	403.7	305.1	305.1	355.4	357.3
None	2.0	6.20	5.10	5.50	5.90		373.9	311.1	313.7	347.5	336.6
	5.0	6.18	5.40	5.56	5.43	6.18	332.7	302.4	306.9	298.7	308.7
	10.0	6.12	5.76	5.80	5.73	5.32	380.7	313.9	303.5	298.7	310.2
	20.0	6.05	6.10	5.93	6.06	6.00	258.3	357.1	306.1	283.7	301.3
		6.09	5.41	5.41	5.40	5.38	167.6	134.3	179.7	169.8	162.8

Element added	Rate of addition	Mat weight				
		Replicate—				
		a	b	c	d	e
	<i>P.p.m.</i>	<i>Mg.</i>	<i>Mg.</i>	<i>Mg.</i>	<i>Mg.</i>	<i>Mg.</i>
Aluminum	1	208.0	140.0	173.9	108.5	158.8
	10	130.7	85.7	82.8	120.5	106.5
	20	124.1	106.3	73.5	133.8	81.3
	40	88.3	72.7	63.6	80.2	60.0
Cadmium	1	260.7	213.6	134.0	196.4	156.9
	10	96.3	106.4	99.4	169.6	124.7
	20	53.7	69.1	77.0	56.1	66.6
	40	38.1	52.0	45.9	41.0	49.3
Cobalt	1	201.5	175.8	178.6	174.4	177.2
	10	25.9	25.3	24.5	22.1	(1)
	20	15.2	7.5	15.6	14.0	10.8
	40	11.7	11.1	10.9	10.7	8.4
Nickel	1	117.2	152.4	160.3	166.1	127.9
	10	37.1	33.3	89.8	115.9	19.5
	20	7.4	6.8	9.1	7.6	6.0
	40	6.4	6.1	6.6	6.6	5.9
Copper	1	163.5	152.2	168.5	167.6	144.6
	10	198.3	99.1	131.6	112.9	136.0
	20	27.0	9.9	13.2	12.5	10.1
	40	4.0	2.1	5.2	4.4	(1)
Zinc	1	479.2	504.4	522.3	586.3	468.7
	10	502.8	563.6	555.8	531.9	550.0
	20	522.7	532.1	577.2	569.9	555.9
	40	492.3	541.9	498.3	530.8	517.9
None		191.2	120.5	139.3	157.1	181.5

<sup>1</sup> Contaminated and discarded.

The studies dealing with the effects of copper, iron, manganese, and zinc upon the growth of *Phymatotrichum omnivorum* were extended to include the effects of aluminum, boron, cobalt, cadmium, fluorine, iodine, lithium, mercury, molybdenum, nickel, and silicon. To the standard nutrient solution, purified by the calcium carbonate treatment, these elements were added to give concentrations of 1, 4, 10, and 20 p. p. m. The addition of these elements singly at these rates failed to give significant increases in mat weight. Definite inhibition of growth to various degrees occurred with the addition of cobalt at 4, 10, and 20 p. p. m., and with nickel at 10 and 20 p. p. m.; aluminum and cadmium showed only a mild inhibition at 4, 10, and 20 p. p. m. The remaining elements appeared to exert slight, if any, influence upon growth of the organism.

To study further and in more detail the action of certain of the elements, an experiment was conducted with the purified solution in which the addition of cobalt, nickel, aluminum, or cadmium was compared with the addition of copper or zinc. The rates of addition were at 1, 10, 20, and 40 p. p. m. The results are summarized in the lower portion of table 1. As usual, copper almost completely inhibited growth at 20 p. p. m., while zinc at all concentrations caused a great increase in mat weight. Both cobalt and nickel were highly toxic to the organism at the two higher concentrations, and a marked depression of growth was displayed at the concentration of 10 p. p. m. As in earlier experiments aluminum and cadmium caused a mild depression of growth at the higher range of concentrations. No significant increases in mat weight occurred, except with the addition of zinc.

#### EFFECT OF ADDING ELEMENTS IN COMBINATION

The effect of adding copper, iron, manganese, and zinc was studied in experiments where all were added to the purified solution or to the unpurified solution, the majority of the experiments being conducted upon the purified solution. The rates of addition ranged from less than 1 p. p. m. to 40 p. p. m. of each element. The lower rates of addition were based upon certain concentrations used by Steinberg (7) in studies with *Aspergillus niger*; that is, copper at 0.12, iron at 0.40, manganese at 0.06, and zinc at 0.28 p. p. m. While increases in mat weight over those obtained in the controls were had by the addition of these elements to the purified solution at these rates, or even at lesser concentrations, it was found that by increasing the rate of addition better growth resulted, as indicated in table 2. Eventually all of these elements were studied in combination at the same rates of addition; that is, at 2, 5, 10, or 20 p. p. m. of each. It was demonstrated that the fungus responded almost equally well in the purified solution to additions of the elements over a range of from less than 1 p. p. m. to 10 p. p. m. The results indicate that, for the range of concentrations used in these experiments, *Phymatotrichum omnivorum* does not have a sharply defined optimum requirement as concerns these elements. With the elements added at the rate of 20 or more p. p. m. growth was depressed directly as the concentration of the elements was increased. It is apparent that when all four elements were present in the solution at 20 p. p. m. of each, the toxic effect of copper (table 1) was largely overcome by the other elements, but at concentrations of 40 p. p. m., almost complete failure of growth occurred.

TABLE 2.—Summary of experiments on the effect of adding copper, iron, manganese and zinc, at various concentrations, upon the growth of *Phymatotrichum omnivorum* in purified solution, incubated at 28° C. for 28 days

Experiments (number)	Rate of addition of—				Flasks	Mean weight of mats	Control (no element added)	
	Cu	Fe	Mn	Zn			Flasks	Mean weight of mats
	P. p. m.	P. p. m.	P. p. m.	P. p. m.	Number	Mg.	Number	Mg.
5	0.12	0.40	0.06	0.28	27	603.0	29	151.1
4	.48	1.60	.24	1.12	24	647.2	23	155.9
9	2.0	2.0	2.0	2.0	43	709.7	41	164.6
8	5.0	5.0	5.0	5.0	37	716.3	36	171.2
4	10.0	10.0	10.0	10.0	18	651.5	18	173.7
2	20.0	20.0	20.0	20.0	7	456.9	8	172.3
2	40.0	40.0	40.0	40.0	8	15.5	8	172.3

Several experiments were conducted in which the four elements in combination were added to the nutrient solution which had not been purified by the calcium carbonate treatment, the range of addition being from less than 1 p. p. m. to 10 p. p. m. of each element. Although increases in mat weight over those obtained in the control flasks resulted from the addition of the elements, the increases were not so great as had occurred with a similar addition to the purified solution. For example, the addition of elements to the unpurified solution yielded mats of 500 mg. or less, whereas a similar addition to the purified solution gave mats weighing 600 to 700 mg. As was to be expected, the controls with the unpurified solution consistently yielded heavier mats than did the controls with the purified solution, indicating the efficiency of the calcium carbonate treatment in the removal of traces of these elements.

It should be observed that in none of the experiments with the purified solution were the mean weights of the controls as low as those reported by Steinberg in his studies with *Aspergillus*. Inasmuch as *Phymatotrichum omnivorum* does not produce a germinable conidial spore, the inoculum used in the present studies consisted of disks of mycelium and adhering agar from agar plate cultures of the organism. Such inoculum, usually 3 to 5 mg. in dry weight, undoubtedly carried traces of the essential elements in the mycelium or in the agar, thus accounting in part for the considerable growth that occurred in the control flasks.

#### FACTORIAL-DESIGN EXPERIMENTS

##### METHOD OF STUDYING INTERACTIONS

In the discussion of the preceding experiments, it was pointed out that, where copper, iron, manganese, and zinc had been added singly to the purified solution, zinc roughly doubled, or more than doubled, the mat weight over that of the control, whereas iron and manganese gave smaller increases, the weights being intermediate between those of zinc and the controls (table 1). Copper gave no consistent increases, although in certain cases it appeared to be slightly beneficial. However, when these elements were used in combination, that is, when all four were present at various levels ranging from less than 1 p. p. m. to 10 p. p. m., the mat weights obtained were approximately four times as great as the controls, indicating the possibility of important interactions. It was therefore deemed advisable to conduct a series of experiments designed to permit a study of these interactions.

Factorial experiments, initiated by Fisher (3), developed by Yates (11), and presented in systematic form by Brandt (1), are particularly suitable for studies of the type mentioned in the preceding paragraph. The essential principles of this type of experiment require that the factors being investigated, in this case copper, iron, manganese, and zinc, be set up in all possible combinations at the given levels of the experiment. In these experiments with 4 elements, 16 combinations or treatments are possible, in which one or more of the elements are present or absent, at a given rate. In the analysis of such an experiment, all combinations of factors could be reestratified for studying all combinations and interactions. Experiments of this type were conducted, the elements being added at the rate of 2, 5, or 10 p. p. m. Throughout the course of these studies precautions were taken to make the several experiments as nearly comparable as possible. The nutrient solution was prepared from stock solutions of the various salts; the purification technique was standard in all; the elements were added at the proper amounts to give the desired concentration; and the inoculum and incubation temperatures were as uniform as possible from experiment to experiment. The various treatments, or combinations of elements, were prepared on the basis of five replicates for a given combination, but losses through contamination or other causes sometimes reduced the number to four by time of harvest. The replicates of a given combination were assigned numbers at random, and were randomized in their location in the incubation chamber. Hence, in analyzing the results of an experiment, no degrees of freedom were lost for stratification of replicates.

#### EFFECT OF ADDING FOUR ELEMENTS SINGLY AND IN COMBINATION

Of a number of such experiments in factorial design, three have been selected as typical of the results obtained at the rates of 2, 5, and 10 p. p. m. respectively. Experiment 91, at 5 p. p. m., was based upon four replicates of each treatment, while experiments 108 and 127, at 10 and 2 p. p. m. respectively, were based upon five replicates of each treatment or combination. The incubation period of experiment 127 was 21 days, while that of the others was, as usual, 28 days.

Separate analyses were made of each experiment. The three experiments were also brought together into a combined analysis, and this is presented along with the separate analyses in tables 3 and 4. The effects of rates of addition of copper, iron, manganese, and zinc, and the interactions involving rates, could be determined only from such a combined analysis. In discussing the results of the three experiments and their separate and combined analyses, the several sources of variance will be dealt with in the order of their appearance in tables 3 and 4.

*Rates.*—It will be noted from the mean square, as well as from the mean mat weights, that the difference between the average mat weight at 2 p. p. m. and at 10 p. p. m. ( $R_1$ ) is enormous. As mentioned above the harvest for the experiment at 2 p. p. m. (experiment 127) was at, 21 days instead of 28 days as in the experiments at 5 and 10 p. p. m. This would account for the consistently lower mat weights obtained at the 2-p. p. m. rate of addition. On the other hand,  $R_2$ , the mean value for rates at 2 and 10 p. p. m., compared with that at 5 p. p. m. does not give significant differences. It should also be borne in mind that since these were separate experiments, rates may be confounded

with such factors as failure to maintain absolutely identical temperatures, differences in vigor of the inoculum, or other factors that are beyond the operator's control. The mean weight at 5 p. p. m. is lower than the value obtained at 10 p. p. m., despite the fact that copper at 10 p. p. m. was somewhat detrimental. In previous experiments it was shown that optimum growth (table 2) occurred at 2 and 5 p. p. m., with little distinction between these rates, and that growth at 10 p. p. m. was only slightly less than that at 2 and 5 p. p. m.

TABLE 3.—Mean values for the effect of copper, iron, manganese, and zinc on growth of *Phycolotrichum omnivorum* (experiments 127, 91, and 108) at rates of 2, 5, and 10 p. p. m. respectively, in purified solutions

Contributor	Mean mat weight at 1—			
	2 p. p. m.	5 p. p. m.	10 p. p. m.	Combined
Cu×Fe×Mn×Zn (× rates):				
Cu absent:				
Fe absent:				
Mn absent:	Mg.	Mg.	Mg.	Mg.
Zn absent:	88	157	134	124
Zn present:	126	321	422	288
Mn present:				
Zn absent:	116	185	282	195
Zn present:	153	381	480	338
Fe present:				
Mn absent:				
Zn absent:	116	197	416	246
Zn present:	516	518	589	543
Mn present:				
Zn absent:	155	227	247	208
Zn present:	522	755	788	684
Cu present:				
Fe absent:				
Mn absent:				
Zn absent:	97	212	184	161
Zn present:	154	339	483	324
Mn present:				
Zn absent:	108	242	240	193
Zn present:	204	433	528	391
Fe present:				
Mn absent:				
Zn absent:	129	243	196	185
Zn present:	500	552	699	607
Mn present:				
Zn absent:	140	261	191	193
Zn present:	558	645	758	655
Rates	234	355	415	
Cu×Fe×Mn (× rates):				
Cu absent:				
Fe absent:				
Mn absent:	107	239	278	206
Mn present:	134	283	386	266
Fe present:				
Mn absent:	316	358	503	395
Mn present:	339	491	518	446
Cu present:				
Fe absent:				
Mn absent:	126	275	334	243
Mn present:	156	347	384	292
Fe present:				
Mn absent:	344	398	447	396
Mn present:	349	453	475	424
Cu×Fe×Zn (× rates):				
Cu absent:				
Fe absent:				
Zn absent:	102	171	208	159
Zn present:	139	351	456	313
Fe present:				
Zn absent:	135	212	332	227
Zn present:	519	637	689	613
Cu present:				
Fe absent:				
Zn absent:	103	227	212	177
Zn present:	179	396	506	358
Fe present:				
Zn absent:	134	252	194	189
Zn present:	559	599	729	631

<sup>1</sup> For convenience, means in this table are given as nearest whole milligram, although tenths of a milligram were recorded and used in the analysis of results.

TABLE 3. — *Mean values for the effect of copper, iron, manganese, and zinc on growth of Phymatotrichum omnivorum (experiments 127, 91, and 108) at rates of 2, 5, and 10 p. p. m. respectively, in purified solutions—Continued*

Contributor	Mean mat weight at—			
	2 p. p. m.	5 p. p. m.	10 p. p. m.	Combined
Cu×Mn×Zn (× rates):				
Cu absent:				
Mn absent:	<i>Mg.</i>	<i>Mg.</i>	<i>Mg.</i>	<i>Mg.</i>
Zn absent	102	177	275	185
Zn present	321	420	506	415
Mn present:				
Zn absent	135	206	265	202
Zn present	337	568	639	511
Cu present:				
Mn absent:				
Zn absent	113	227	190	173
Zn present	357	446	591	466
Mn present:				
Zn absent	124	251	216	193
Zn present	381	549	643	523
Fe×Mn×Zn (× rates):				
Fe absent:				
Mn absent:				
Zn absent	93	184	159	142
Zn present	140	330	453	306
Mn present:				
Zn absent	112	213	261	194
Zn present	178	417	509	364
Fe present:				
Mn absent:				
Zn absent	122	220	306	216
Zn present	538	535	644	675
Mn present:				
Zn absent	147	244	219	201
Zn present	540	700	773	669
Cu×Fe (× rates):				
Cu absent:				
Fe absent	121	261	332	236
Fe present	327	424	510	420
Cu present:				
Fe absent	141	311	359	267
Fe present	347	425	461	410
Cu×Mn (× rates):				
Cu absent:				
Mn absent	211	299	390	300
Mn present	236	387	452	356
Cu present:				
Mn absent	235	336	390	319
Mn present	253	400	429	358
Cu×Zn (× rates):				
Cu absent:				
Zn absent	119	191	270	193
Zn present	329	494	572	463
Cu present:				
Zn absent	119	239	203	183
Zn present	369	497	617	494
Fe×Mn (× rates):				
Fe absent:				
Mn absent	116	257	306	224
Mn present	145	315	385	279
Fe present:				
Mn absent	330	378	475	395
Mn present	344	472	496	435
Fe×Zn (× rates):				
Fe absent:				
Zn absent	102	199	210	168
Zn present	159	373	481	335
Fe present:				
Zn absent	135	232	263	208
Zn present	539	618	709	622
Mn×Zn (× rates):				
Mn absent:				
Zn absent	107	202	233	179
Zn present	339	433	548	441
Mn present:				
Zn absent	130	229	240	197
Zn present	359	558	641	517
Cu (× rates):				
Cu absent	224	343	421	328
Cu present	244	308	410	339
Fe (× rates):				
Fe absent	131	286	345	252
Fe present	337	425	486	415



TABLE 3.—Mean values for the effect of copper, iron, manganese, and zinc on growth of *Phymatotrichum omnivorum* (experiments 127, 91, and 108) at rates of 2, 5, and 10 p. p. m. respectively, in purified solutions—Continued

Contributor	Mean mat weight at—			
	2 p. p. m.	5 p. p. m.	10 p. p. m.	Combined
Mn (X rates):	<i>Mg.</i>	<i>Mg.</i>	<i>Mg.</i>	<i>Mg.</i>
Mn absent	223	317	390	310
Mn present	244	394	441	357
Zn (X rates):				
Zn absent	119	215	236	188
Zn present	349	496	595	479

TABLE 4.—Analysis of variance for effects of copper, iron, manganese, and zinc on growth of *Phymatotrichum omnivorum* in experiments 127, 91, and 108, at rates of 2, 5, and 10 p. p. m. respectively

Variance	2 p. p. m.		5 p. p. m.		10 p. p. m.		Combined analysis	
	D. F.	Mean square <sup>1</sup>	D. F.	Mean square <sup>1</sup>	D. F.	Mean square <sup>1</sup>	D. F.	Mean square <sup>1</sup>
Rates: <sup>2</sup>								
R <sub>1</sub>							1	*1,319,397
R <sub>2</sub>							1	*43,390
Cu	1	*7,950	1	10,490	1	2,503	1	6,108
R <sub>1</sub> ×Cu							1	9,688
R <sub>2</sub> ×Cu							1	5,156
Fe	1	*849,153	1	*307,706	1	*394,735	1	*1,494,930
R <sub>1</sub> ×Fe							1	*42,988
R <sub>2</sub> ×Fe							1	*13,675
Mn	1	*9,008	1	*92,576	1	*50,305	1	*124,887
R <sub>1</sub> ×Mn							1	8,369
R <sub>2</sub> ×Mn							1	*18,633
Zn	1	*1,064,334	1	*1,256,053	1	*2,567,399	1	*4,722,630
R <sub>1</sub> ×Zn							1	*162,818
R <sub>2</sub> ×Zn							1	2,338
Cu×Fe	1	5	1	9,754	1	*28,861	1	*24,230
R <sub>1</sub> ×Cu×Fe							1	*14,051
R <sub>2</sub> ×Cu×Fe							1	339
Cu×Mn	1	264	1	2,349	1	2,573	1	4,346
R <sub>1</sub> ×Cu×Mn							1	594
R <sub>2</sub> ×Cu×Mn							1	245
Cu×Zn	1	*7,962	1	7,867	1	*62,658	1	*24,192
R <sub>1</sub> ×Cu×Zn							1	12,974
R <sub>2</sub> ×Cu×Zn							1	*41,310
Fe×Mn	1	1,130	1	5,262	1	16,574	1	3,394
R <sub>1</sub> ×Fe×Mn							1	4,524
R <sub>2</sub> ×Fe×Mn							1	*15,048
Fe×Zn	1	*603,208	1	*178,686	1	*153,186	1	*853,708
R <sub>1</sub> ×Fe×Zn							1	*74,218
R <sub>2</sub> ×Fe×Zn							1	7,094
Mn×Zn	1	29	1	*39,467	1	*36,146	1	*46,983
R <sub>1</sub> ×Mn×Zn							1	*19,058
R <sub>2</sub> ×Mn×Zn							1	9,599
Cu×Fe×Mn	1	527	1	11,265	1	6,151	1	556
R <sub>1</sub> ×Cu×Fe×Mn							1	5,139
R <sub>2</sub> ×Cu×Fe×Mn							1	12,248
Cu×Fe×Zn	1	7	1	4,451	1	21,783	1	2,930
R <sub>1</sub> ×Cu×Fe×Zn							1	10,504
R <sub>2</sub> ×Cu×Fe×Zn							1	12,807
Cu×Mn×Zn	1	1,180	1	1,617	1	17,173	1	6,285
R <sub>1</sub> ×Cu×Mn×Zn							1	*13,679
R <sub>2</sub> ×Cu×Mn×Zn							1	7
Fe×Mn×Zn	1	2,148	1	6,854	1	*86,284	1	*36,903
R <sub>1</sub> ×Fe×Mn×Zn							1	*67,828
R <sub>2</sub> ×Fe×Mn×Zn							1	554
Cu×Fe×Mn×Zn	1	123	1	8,600	1	*43,806	1	*32,890
R <sub>1</sub> ×Cu×Fe×Mn×Zn							1	*19,674
R <sub>2</sub> ×Cu×Fe×Mn×Zn							1	25
Error	64	804	48	1,897	64	3,202	176	1,974
Total	79	32,893	63	32,286	79	46,774	223	43,455
Mean square required for significance:								
Odds 99 : 1		5,696		13,713		22,669		13,344
Odds 19 : 1		3,218		7,701		12,808		7,679

<sup>1</sup> Asterisk (\*) indicates that contributor is highly significant (odds 99 : 1). Calculations for determining significance were based on *F* values and *t* values taken from Snedecor's table (8).

<sup>2</sup> R<sub>1</sub> represents comparison of rates at 2 p. p. m. and 10 p. p. m.; R<sub>2</sub> represents mean value for rates at 2 and 10 p. p. m. as compared with 5 p. p. m.

*Copper.*—The presence of copper gave no highly significant increase over the values obtained in its absence except at 2 p. p. m., where the *F* value slightly exceeded the requirement for high significance.

*Iron.*—The presence of iron more than doubled the mat weight secured in the cultures from which iron was absent at 2 p. p. m., and was roughly one-half greater at both 5 and 10 p. p. m. The difference, of course, is highly significant at all rates.

*Manganese.*—The presence of manganese also gave a significant increase over the results obtained in its absence at all rates. The percentage of increase however, was relatively much smaller than that for iron.

*Zinc.*—At all rates the presence of zinc resulted in mat weights that exceeded by at least 100 percent those obtained in the absence of zinc.

*Interactions.*—It is exceedingly difficult to visualize interactions. Hence it seems desirable to supplement the tabular data with the scheme shown in figure 1 for presenting graphically the results obtained for experiment 108 at 10 p. p. m., in which significant interactions were obtained more frequently than at 5 and 2 p. p. m.<sup>4</sup>

*Interaction of Cu × Fe.*—Interaction was significant at 10 p. p. m., but not at 2 or 5 p. p. m. The means for the presence of copper and iron at 2 and 5 p. p. m. barely exceeded the values for iron alone. At 10 p. p. m., the results with iron alone appreciably exceeded those obtained in the presence of both iron and copper. The presence of copper alone gave but a slight increase over the values obtained in the absence of both copper and iron at 10 p. p. m., but more important increases at 2 and 5 p. p. m. This is illustrated in figure 1, where it appears that the presence of copper gave some increase in the absence of iron, but that copper was decidedly harmful when iron was present.

*Interaction of Cu × Mn.*—Significance was not obtained for interaction of copper and manganese at any of the three rates. It also appears from the means that the combinations are little better or poorer than might be anticipated by the simple addition of beneficial effects due to copper and manganese, as shown in the discussion above, when the results are analyzed for the presence or absence of either one of these singly. The graph shows that the addition of copper at 10 p. p. m. was without effect in the absence of manganese and was somewhat harmful when manganese was present.

*Interaction of Cu × Zn.*—At the three rates the mean weights in the presence of both zinc and copper slightly exceeded those obtained with zinc alone. Copper alone gave values identical with those obtained in the absence of both copper and zinc at 2 p. p. m., a slight increase at 5, and an appreciable decrease at 10. It will be noted that high significance for this interaction was reached at 2 and at 10 p. p. m., and that the interaction with  $R_2$  was significant. This is somewhat unusual in that generally the results for 5 p. p. m. follow the linear component for 2 and 10 p. p. m. From the graph it will be noted that copper at 10 p. p. m. was distinctly harmful in the absence of zinc, but that it was beneficial when zinc was present. At 2 p. p. m. the results were similar to the extent that copper was beneficial in the presence of zinc. At 5 p. p. m. copper was beneficial in the absence and without effect in the presence of zinc.

<sup>4</sup> The scheme for graphic representation of comparisons, used in figures 1 and 2, was furnished by H. D. Barker.

*Interaction of Fe  $\times$  Mn.*—Iron and manganese show no significant interactions at 2, 5, or 10 p. p. m. However, it will be noted that the interaction  $R_2 \times Fe \times Mn$  reaches significance. At 2 p. p. m. the presence of manganese gives but slight increase over its absence when iron is either present or absent, the increase being relatively greater when iron is absent. At 10 p. p. m. the results are similar in that the presence of manganese is almost without effect when iron is present,

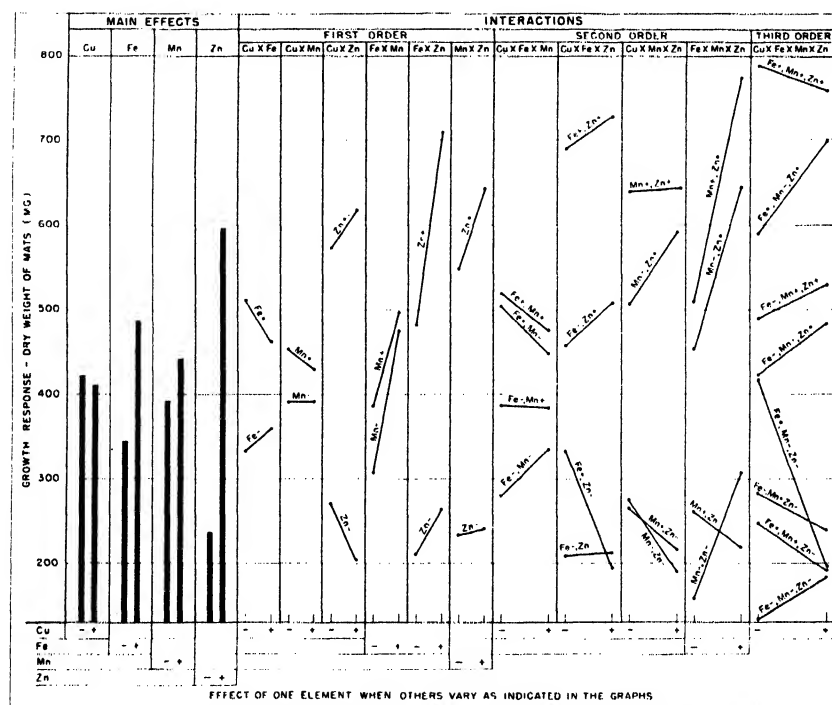


FIGURE 1.—Graphic representation of comparisons for main effects and interactions for experiment 108. The significance of an interaction is indicated by the degree of failure of the curves to extend in a parallel direction. The angle of convergence furnishes a measure for the relative importance of the various interactions, a wide angle being associated with a large contribution. The dry weight of mats produced was used to measure the growth response of *Phycomatrichum omnivorum* to the presence or absence indicated by plus (+) or minus (−) signs, at 10 p. p. m., of four elements in purified solutions.

and based upon comparative weights in the experiments at 2 and 10 p. p. m., the presence of manganese is of approximately equal value in the absence of iron. At 5 p. p. m. the results differ materially in that manganese is highly beneficial in the presence of iron, and in the absence of iron gives about the same proportional increase as occurred at the other rates.

*Interaction of Fe  $\times$  Zn.*—This interaction is significant at all levels. At 2 p. p. m. the combined effect of iron and zinc gives about three and one-half times the value obtained in the presence of either iron or zinc

when the other member of the combination is absent. At 5 p. p. m. the mean weight with both elements present is not quite double that of zinc alone, but is nearly three times as large as that of iron alone, and the latter only slightly exceeds the values obtained when both are absent. At 10 p. p. m. the relationships parallel very closely those obtained at 5 p. p. m. Thus at 2 p. p. m. the results are materially different from those at 5 and 10 p. p. m. It appears likely that most of this difference is taken out with the interaction of  $R_1$ , which is highly significant, so that  $R_2$  scarcely reaches the 5-percent requirement.

*Interaction of  $Mn \times Zn$ .*—This interaction is significant at 5 and 10 p. p. m. At 2 p. p. m. the mean value in the presence of both elements only slightly exceeds that in the presence of zinc alone, and in the presence of manganese alone the mean value is little more than that obtained in the absence of both elements. At 5 p. p. m. the mean weight of mats obtained in the presence of manganese and zinc exceeds by as much as 25 percent the mean weight with zinc alone, while with manganese present alone, the mean weight is only slightly better than with both manganese and zinc absent, as was the case at 2 p. p. m. At 10 p. p. m., the value obtained with both manganese and zinc present appreciably exceeds that obtained with zinc alone, and again there is but slight difference between the values with manganese present alone and with both absent.

*Second- and third-order interactions.* For the second-order interaction, in which rates are not involved, no significant differences are obtained except for  $Fe \times Mn \times Zn$  at 10 p. p. m. and in the combined analysis. At all three rates, the mean weight with iron, manganese, and zinc present shows that this is a particularly favorable combination. The mean weight with iron and manganese present and zinc absent does not exceed one-third of the preceding value. The value for iron and zinc about equals that for all three present at 2 p. p. m. and is about 25 percent less at 5 and 10 p. p. m. Manganese and zinc both present is the next best combination, although at 2 p. p. m. it only slightly exceeds some of the less favorable combinations. Zinc alone does not appear in favorable light for 2 p. p. m., but at 5 and 10 p. p. m. it is appreciably better than iron and is only slightly below manganese plus zinc. The interaction of  $Cu \times Fe \times Mn \times Zn$  reaches high significance at 10 p. p. m. Figure 1 shows that in the absence of zinc the beneficial effects of iron are nullified when manganese or copper is added or when both are present, whereas, in the presence of zinc, iron not only reacts very favorably, but the additional presence of manganese or copper is beneficial although copper is slightly harmful if all three of the others are present. In considering the means for the interaction of  $Cu \times Fe \times Mn \times Zn$ , it should be recalled that each of the means given here represents the results from five replicates at 2 and 10 p. p. m. and from four replicates at 5 p. p. m., and that the 2 p. p. m. experiment was harvested after 21 days' incubation, while the other experiments were harvested at 28 days.

EFFECT OF VARYING ONE ELEMENT AT A TIME, WITH THREE  
HELD CONSTANT

To supplement certain of the results obtained in the preceding factorial experiments, four experiments, designated as 114, 115, 116,

and 117, were conducted in which the rate of one element was varied while the three other elements were held at a constant level of either 2 or 5 p. p. m. The element under consideration in a given experiment was studied as to absence or presence at 0, 2, 5, 10, 20, and 30 p. p. m., and each experiment was further divided to include two types of solution, purified and unpurified. For example, in experiment 114, manganese was the variable element, being used at 0, 2, 5, 10, 20, or 30 p. p. m. in both purified and unpurified solutions. Each kind of solution had received the three other elements (copper, iron, and zinc) at the rates of 2 and 5 p. p. m. of each. The factors under consideration in this experiment were kind of solution (2), level of three other elements (2), and rate of Mn application (6), giving a  $2 \times 2 \times 6$  factorial, 24 treatments or combinations being required. As usual, the treatments were prepared on the basis of five flasks for each solution combination. In two of the experiments no contaminations occurred and all five replicates were included in the analyses. In the other two experiments each had one contaminated flask in one or more of the series so that only four replicates were included in the analyses, the fifth replicate, when available, being chosen at random and discarded.

From the outline just given of experiments 114 to 117 it will be seen that one of the objects was to test purified in comparison with unpurified solutions. As stated under discussion of methods, the formula for the unpurified solution requires that ferric chloride ( $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ) be added to the equivalent of 0.46 p. p. m. of iron. Hence, since it was impossible to test the standard unpurified solution at 0 p. p. m. for iron, the mean values of the four replicates obtained on the unpurified solution in experiment 116 are inserted in table 5 in their appropriate place, in parentheses. They are not included in the analysis of variance for experiment 116 in table 5, nor were these values used in obtaining the other mean values that are recorded for this experiment. It should also be borne in mind that in the other experiments (114, 115, and 117), where it is stated that iron and other elements are held constant at 2 or 5 p. p. m., this actually means that 2.46 and 5.46 p. p. m. are the correct values for iron.

Although these are separate experiments and in some respects are not directly related to each other, the results will be discussed on the basis of sources of variance, and the interpretations offered are based on the mean values and the analyses of variance of the respective experiments, as presented in table 5.

*Kind of solution.*—It will be seen from the mean values in table 5 that the purified solution gave very much higher results than the unpurified solution in all four experiments. These differences are highly significant.

*Level of three elements held constant.*—There was little difference in the results between 2 p. p. m. and 5 p. p. m. except where copper was the variable element (experiment 117), in which case the value at 5 p. p. m. was distinctly lower than at 2 p. p. m.

TABLE 5.- *Mean values and analysis of variance of mat weights of Phymatotrichum omnivorum in experiments in which one element was varied while others were held constant at 2 or 5 p. m. on two types of solution, with incubation at 28° C. for 28 days*

## COPPER VARIABLE (EXPERIMENT 117)

Variance	Mean mat weight					Analysis of variance	
	Mean		Difference required for significance (odds 99:1)			D. F.	Mean square <sup>1</sup>
	<i>Mg.</i>		<i>Mg.</i>				
Kinds of solution						1	*1,534,923
Purified	726.1		50.2				
Unpurified	478.3					1	*126,764
Levels for 3 elements			50.2				
2 p. p. m.	637.8						
5 p. p. m.	566.6						
Rates of Cu			79.3			4	*182,657
0 p. p. m.	719.8						
2 p. p. m.	639.9						
5 p. p. m.	640.1						
10 p. p. m.	530.3						
20 p. p. m.	481.0						
Kinds × levels						1	7,566
Mean mat weight with 3 elements at—							
	2 p. p. m.		5 p. p. m.				
Kinds of solution:	<i>Mg.</i>		<i>Mg.</i>				
Purified	753.0		699.2				
Unpurified	522.6		434.0				
Kinds × rates						4	*69,406
Mean mat weight with Cu at—							
	0	2	5	10	20		
	p. p. m.	p. p. m.	p. p. m.	p. p. m.	p. p. m.		
	<i>Mg.</i>	<i>Mg.</i>	<i>Mg.</i>	<i>Mg.</i>	<i>Mg.</i>		
Kinds of solution:	753.1	773.7	796.0	719.6	588.2		
Purified	686.4	506.1	484.2	341.0	373.9		
Unpurified						4	*63,006
Levels × rates							
Levels at							
2 p. p. m.	705.3	630.3	668.6	580.0	604.9		
5 p. p. m.	734.3	649.5	611.6	480.6	357.1		
Kinds × levels × rates						4	21,536
Purified, levels at --							
2 p. p. m.	745.0	746.7	828.4	708.3	736.6		
5 p. p. m.	761.3	800.6	763.5	730.9	439.7		
Unpurified, levels at--							
2 p. p. m.	665.6	513.8	508.8	451.7	473.2		
5 p. p. m.	707.3	498.3	459.7	230.2	274.6		
Error						80	9,037
Total						99	76,734

IRON VARIABLE (EXPERIMENT 116) <sup>2</sup>

Variance	Mean mat weight		Analysis of variance	
	Mean	Difference required for significance (odds 99:1)	D. F.	Mean square <sup>1</sup>
Purified solution:	<i>Mg.</i>	<i>Mg.</i>		
Levels for 3 elements		25.7	1	2,844
2 p. p. m.	726.7			
5 p. p. m.	742.1			
Rates of Fe		44.5	5	*78,927
0 p. p. m.	533.5			
2 p. p. m.	759.0			
5 p. p. m.	785.6			
10 p. p. m.	785.1			
20 p. p. m.	785.6			
30 p. p. m.	757.6			
Levels × rates			5	1,586

See footnotes at end of table.

TABLE 5.—Mean values and analysis of variance of mat weights of *Phymatotrichum omnivorum* in experiments in which one element was varied while others were held constant at 2 or 5 p. m. on two types of solution, with incubation at 28° C. for 28 days—Continued

IRON VARIABLE (EXPERIMENT 116)—Continued											
Variance	Mean mat weight with Fe at—						Analysis of variance				
	0 p. p. m.	2 p. p. m.	5 p. p. m.	10 p. p. m.	20 p. p. m.	30 p. p. m.	D. F.	Mean square			
Purified solution—Continued.											
Levels for 3 elements:											
2 p. p. m.	Mg. 545.9	Mg. 745.9	Mg. 777.5	Mg. 789.8	Mg. 759.7	Mg. 741.4					
5 p. p. m.	521.1	772.1	793.8	780.4	811.5	773.8					
(Unpurified solution):											
(Levels at—											
2 p. p. m.)	(503.6)	(483.4)	(458.6)	(454.3)	(469.5)	(485.8)					
5 p. p. m.)	(470.7)	(457.1)	(437.8)	(454.1)	(453.7)	(431.0)					
Error							36	1,067			
Total							47	9,443			
MANGANESE VARIABLE (EXPERIMENT 114)											
Variance	Mean mat weight				Analysis of variance						
	Mean		Difference required for significance (odds 99:1)		D. F.	Mean square <sup>1</sup>					
	Mg.		Mg.								
Kinds of solution			26.4		1	*2,206,417					
Purified	806.3										
Unpurified	503.1										
Levels for 3 elements:			26.4		1	3,360					
2 p. p. m.	662.2										
5 p. p. m.	647.3										
Rates of Mn			45.7		5	*10,040					
0 p. p. m.	641.9										
2 p. p. m.	660.6										
5 p. p. m.	648.1										
10 p. p. m.	616.6										
20 p. p. m.	673.6										
30 p. p. m.	687.7										
Kinds × levels					1	*71,215					
Mean mat weight with 3 elements at—											
2 p. p. m.					5 p. p. m.						
Mg.					Mg.						
Kinds of solution:	786.6		826.1		5	*15,702					
Purified	537.9		468.4								
Unpurified											
Kinds × rates											
Mean mat weight with Mn at—											
0                      2                      5                      10                      20                      30											
p. p. m.                      p. p. m.                      p. p. m.                      p. p. m.                      p. p. m.                      p. p. m.											
Mg.                      Mg.                      Mg.                      Mg.                      Mg.                      Mg.											
Kinds of solution:	827.1	826.4	766.5	726.1	826.4	865.7	5	626			
Purified	456.7	494.8	529.7	507.1	520.9	509.6					
Unpurified											
Levels × rates											
Levels at—											
2 p. p. m.	651.4	665.1	657.4	633.7	679.8	685.9					
5 p. p. m.	632.3	656.1	638.8	599.5	667.5	689.4					
Kinds × levels × rates							5	*9,249			
Purified:											
Levels at—											
2 p. p. m.	841.5	803.9	765.6	720.4	771.4	816.9					
5 p. p. m.	812.6	849.0	767.4	731.8	881.4	914.5					
Unpurified:											
Levels at—											
2 p. p. m.	461.3	526.4	549.2	547.0	588.2	555.0					
5 p. p. m.	452.1	463.2	510.2	467.3	453.6	464.3					
Error							72	2,382			
Total							95	27,712			

See footnotes at end of table.





caused a considerable drop, and 20 p. p. m. a further drop; copper at 30 p. p. m. behaved in such an erratic manner that the results were not considered reliable, and accordingly they have not been included. For iron (experiment 116), which included only the purified solution, the result obtained in its presence was distinctly superior to that obtained in its absence. There was little difference between rates ranging from 2 to 30 p. p. m. The presence of manganese (experiment 114) at various rates was slightly superior to its absence, except at 10 p. p. m. The differences between rates barely reached significance at the 1-percent requirement. For zinc there was a remarkable difference between presence and absence, values with the latter being about one-third of those with the former. No appreciable difference occurred with zinc in rates from 2 to 30 p. p. m.

*Kind*×*level*.—In experiment 117, with copper variable, the purified solution at 2 p. p. m. was distinctly superior to the 5 p. p. m.; the same relationship between 2 and 5 p. p. m. existed in the unpurified solution. For experiment 114, with manganese variable, 5 p. p. m. was appreciably better than 2 p. p. m. in the purified solution. The reverse was true of the unpurified solution, a highly significant *F* value for *kind*×*level* being found in experiment 114 (see table 5). There was little difference between the results at 2 and 5 p. p. m. in the purified solution for zinc, although in the unpurified solution 2 p. p. m. was slightly better. The interaction was highly significant in this experiment (115).

*Kind*×*rate*.—At 0 to 5 p. p. m., copper in the purified solution gave very excellent growth; at 10 p. p. m. growth was only slightly inferior; but at 20 p. p. m. it was decidedly poorer. In the unpurified solution the amount of growth in general decreased with increasing concentrations of copper. It is important to note again the very considerable increase in growth when there was no copper in the unpurified solution. The significant interaction is probably explained by the fact that depression of growth with the increasing concentrations of copper commenced sooner and progressed more rapidly in the unpurified solution than in the purified. Manganese gave very fine growth in the purified solution at all rates. In the unpurified solution, for which mat weight was less than two-thirds that of the purified solution, concentration seemed also to have little effect, except that growth averaged about 50 mg. less when manganese was absent than when it was present. This interaction was significant at the 1-percent requirement. For experiment 115, with zinc variable, growth was very poor in both the purified and the unpurified solution in the absence of zinc. In the purified solution the average mat weight tended to increase with increasing concentration, whereas in the unpurified solution the rate of growth decreased at a fairly regular rate with increasing concentration. The interaction was significant.

*Level*×*rate*.—In experiment 117, the mat weights were decidedly better in the absence of copper than in its presence for both the 2 p. p. m. and the 5 p. p. m. levels of other elements. Increasing concentrations of copper from 2 to 20 p. p. m. seemed to be without material effect at the 2 p. p. m. level, whereas at the 5 p. p. m. level of other elements the decrease in mat weight was fairly rapid with increase of concentrations. In the purified solution (experiment 116), which is the only solution included for iron, rate of application removed most

of the variance. Interaction was not significant. For manganese the condition was very similar to that for iron, rate removing most of the variance. For zinc, optimum growth was apparently reached at the highest concentrations when the level of other elements was held at 2 p. p. m., although growth with zinc at 30 p. p. m. was not very much greater than with zinc at 2 p. p. m. When the other elements were held at 5 p. p. m., the optimum appeared to be reached at 2 and 5 p. p. m. of zinc, 10, 20, and 30 p. p. m. of zinc giving lower results which did not differ greatly from one another.

*Kind  $\times$  level  $\times$  rate.*—Although the highest order interaction did not reach significance at the 1-percent requirement for experiment 117, several interesting comparisons are apparent. With copper absent in the unpurified solution and the other elements at 5 p. p. m., the mean mat weight for five replicates was over 700 mg., and with the other elements present at 2 p. p. m. (and copper absent) the average was only about 40 mg. less. The decrease with additional amounts of copper was quite rapid in the unpurified solution when the other elements were held constant at 5 p. p. m. One other feature of interest regarding copper is that, when the other elements were present at 2 p. p. m. in the purified solution, as much as 20 p. p. m. of copper seemed to do little harm. When the other elements were at 5 p. p. m. in the purified solution the depression in mat weight was quite sharp at 20 p. p. m. of copper.

Although the results for iron in the unpurified solution were not included in the means or the analysis shown in table 5 (experiment 116), the values are included in parentheses because they appeared to be of interest in connection with the copper and iron relationship. These results are interpreted to indicate that when iron is added to the amount of only 0.46 p. p. m. in the unpurified solution the growth obtained is better than when iron is present in larger amounts. Increasing amounts of iron in purified solution appeared to do no harm. These results indicate some unexplained incompatibility of copper and iron in the unpurified solution. In experiment 108 it was shown that slightly deleterious effects occurred when copper and iron at 10 p. p. m. were present, even in the purified solution.

Interactions were significant for manganese and zinc (experiments 114 and 115). In the purified solution manganese did not appear to be beneficial, nor did increasing amounts of manganese appear to do harm when the other elements were at 2 or 5 p. p. m. The highest mean value obtained was with manganese at 30 p. p. m. and the other elements at 5 p. p. m. In the unpurified solution, growth in the presence of manganese was somewhat better than in its absence when the other elements were at 2 p. p. m. The presence or absence of manganese had little appreciable effect upon growth in the unpurified solution with the other elements at 5 p. p. m. Increasing amounts of zinc up to 30 p. p. m. appeared to be beneficial in the purified solution with other elements at 2 p. p. m.; with other elements at 5 p. p. m. optimum growth with zinc occurred at 2 to 5 p. p. m. In the unpurified solution with other elements at 2 p. p. m., the optimum concentration of zinc was 2 p. p. m. with rather sharp decreases above 10 p. p. m. A similar condition existed with the other elements at 5 p. p. m. In all four treatments, where zinc was absent growth was poor.

## EFFECT OF VARYING IRON AND ZINC WITH MANGANESE HELD CONSTANT

Several of the problems involved in determining the best type of medium for optimum growth of the root rot organism were solved in the foregoing experiments (114-117), when it became evident that it would not be necessary to resort to the laborious process of purification since excellent growth could be obtained on the unpurified solution when copper was omitted. Under the heading "Kind of solution" (p. 149), it was pointed out that the mean for the purified solution ex-

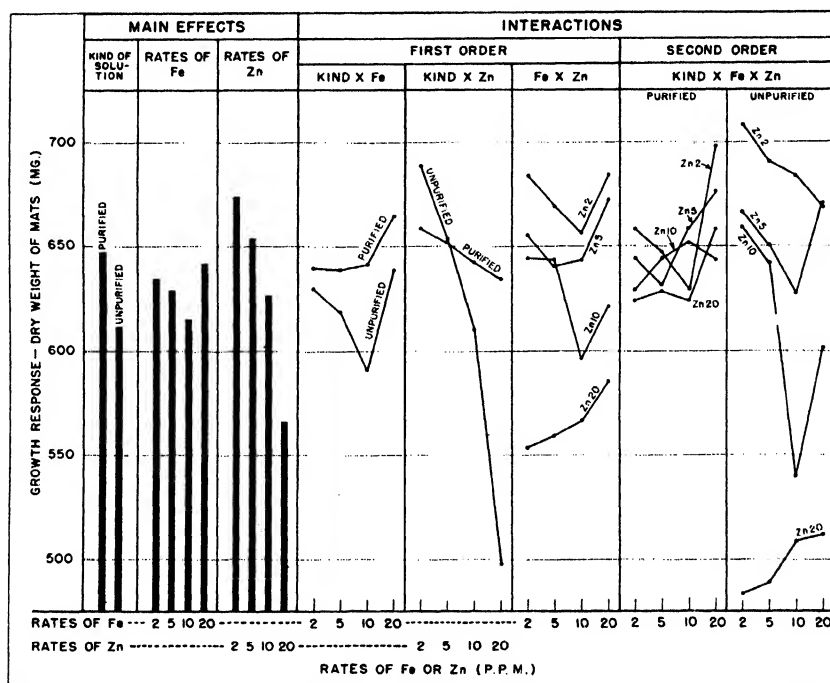


FIGURE 2.—Graphic representation of comparisons for main effects and interactions for experiment 119. Curves representing second-order comparisons are divided into two groups and compared side by side instead of being superimposed on each other as in figure 1. Nevertheless, curves for the purified solution are nearly on top of each other as shown. The dry weight of mats produced was used to measure the response of *Phytophthora omnivora* to varying rates of iron and zinc in purified and unpurified solutions. Copper was omitted and manganese was held constant at 2 p. p. m.

ceeded by at least 50 percent that for the unpurified solution. Somewhat similar relationships prevailed in all previous experiments. The results with copper absent from the unpurified solution indicated how this might be corrected. It was demonstrated that the rate of manganese application was of little importance and that the presence of manganese gave results only slightly superior to its absence. Hence in the present experiment copper was omitted and manganese was held constant at 2 p. p. m. Iron and zinc were used in a factorial design at four levels, 2, 5, 10, and 20 p. p. m. The unpurified solution in this experiment differed from that in the preceding experiments (114-117) in that ferric chloride ( $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ) was omitted in the

preparation of the solution, hence iron would be present only as impurities or as added later in the various treatments. As in the preceding experiment, five flasks of each treatment were prepared but only four were considered in the analysis, the fifth being discarded in each case by random choice if no contaminations developed. The results of this experiment are presented in table 6 and in figure 2.

*Kind of solution.*—The growth in purified solution exceeded that in the unpurified solution by less than 35 mg. However, since these means were derived from 64 replicates each, this difference reaches high significance.

TABLE 6.—*Effect of varying iron and zinc at four rates, with manganese held constant (experiment 119)*

Variance	Mean mat weight		Analysis of variance	
	Mean	Difference required for significance (odds 99:1)	D. F.	Mean square <sup>1</sup>
Kinds of solution	<i>Mg.</i>	<i>Mg.</i>		
Purified	646.59	16.94	1	*37,565
Unpurified	612.32			
Rates of iron application		23.95	3	3,798
Rates of zinc application		23.95	3	*69,349
	Mean mat weight with Fe variable	Mean mat weight with Zn variable		
	<i>Mg.</i>	<i>Mg.</i>		
Rates of iron or zinc:				
2 p. p. m.	633.93	672.88		
5 p. p. m.	627.61	652.95		
10 p. p. m.	615.31	626.20		
20 p. p. m.	640.97	565.79		
Kinds of solution × Rates of Fe			3	4,117
Kinds of solution × Rates of Zn			3	*41,581
Kinds × Fe or Zn:				
Purified, rate at—				
2 p. p. m.	638.95	658.17		
5 p. p. m.	637.60	652.58		
10 p. p. m.	640.80	641.93		
20 p. p. m.	668.99	633.68		
Unpurified, rate at—				
2 p. p. m.	628.90	687.69		
5 p. p. m.	617.62	653.32		
10 p. p. m.	589.83	610.48		
20 p. p. m.	612.95	497.91		
Rates of Fe × Rates of Zn			9	1,664
	Mean mat weight with zinc at—			
	2 p. p. m.	5 p. p. m.	10 p. p. m.	20 p. p. m.
	<i>Mg.</i>	<i>Mg.</i>	<i>Mg.</i>	<i>Mg.</i>
Fe at—				
2 p. p. m.	682.84	655.34	643.03	553.50
5 p. p. m.	668.58	640.30	643.00	558.56
10 p. p. m.	656.31	643.14	595.69	566.11
20 p. p. m.	683.80	673.01	622.09	584.99
Kind × Fe × Zn				
Purified, Fe at—				
2 p. p. m.	658.08	644.28	629.18	624.28
5 p. p. m.	647.48	630.83	644.28	627.83
10 p. p. m.	628.98	650.08	650.98	624.18
20 p. p. m.	698.15	676.13	643.28	658.43
Unpurified, Fe at—				
2 p. p. m.	707.60	660.40	658.88	482.73
5 p. p. m.	689.68	649.78	641.73	489.30
10 p. p. m.	683.65	627.20	540.40	508.05
20 p. p. m.	669.45	669.90	600.90	511.65
Error				
			96	1,331
Total			127	4,413

<sup>1</sup> Asterisk (\*) indicates that *F* value obtained is highly significant (odds 99:1).

*Rates of Fe application.*—The mean mat weights with iron at concentrations of 2, 5, and 20 p. p. m. were not significantly different. For some reason difficult to explain on the basis of available data, 10 p. p. m. gave comparably low yields, whereas 20 p. p. m. gave an average mat weight which slightly exceeded that obtained at 2 p. p. m.

*Rates of Zn application.*—The optimum concentration for zinc was clearly at 2 p. p. m. in the combined means obtained from the different kinds of solution, and from various combinations with iron. Increasing concentration resulted in steady decreases.

*Kind of solution  $\times$  Fe.*—With iron it will be noted that the purified solution gave a better performance than the unpurified solution, although at 2 p. p. m. the difference was small. In the purified solution, there was little difference between 2, 5, and 10 p. p. m. while 20 p. p. m. gave a considerably higher mean mat weight. In the unpurified solution, results with iron at 2 p. p. m. appeared to be optimum, with iron at 5 p. p. m. somewhat lower, and with iron at 10 p. p. m. distinctly inferior. Here again the peculiar increase with 20 p. p. m. mentioned above will be noted. Since highly significant interactions were obtained in this experiment and since one of the primary objects was to clarify the intricate relationships of these two most important elements (iron and zinc), it is felt that the preceding tables and analyses of variance could be more easily interpreted by the aid of the graphs presented in figure 2.

*Kind of solution  $\times$  Zn.*—In the purified solution the different rates of zinc were comparatively unimportant. However, there was a slight downward trend with increasing concentration. In the unpurified solution, the values with zinc at 2 p. p. m. exceeded all values obtained in the purified solution, and there was a very rapid and regular decrease from 2 to 10 p. p. m.; from 10 to 20 p. p. m. the decrease was even greater. This resulted in a very high  $F$  value for interaction.

*Fe  $\times$  Zn.*—At all concentrations of iron, 2 p. p. m. of zinc gave the best average results, and increasing concentrations of zinc resulted in fairly regular average decreases. Or, considering the curves for zinc, it will be noted that 2 and 20 p. p. m. of iron gave the best general results, except when zinc was at 20 p. p. m., with a particularly unfavorable combination for zinc at 2 and 10 p. p. m. However, since these differences were brought out with rates of application, there is no significant interaction for rates of iron  $\times$  rates of zinc.

*Kind of solution  $\times$  Fe  $\times$  Zn.*—This is not a significant interaction. The behavior of the four rates of iron application respond very much in the same manner with increasing concentration of zinc, particularly in the unpurified solution. In the purified solution, the differences due to increasing concentration of zinc are small and somewhat irregular, but again their trend is downward.

From a practical point of view, the most important features of the above experiment were as follows: (1) The results suggested in experiments 114 to 117, that the unpurified solution would serve satisfactorily for optimum growth, were confirmed; actually the highest mat weight recorded was with the unpurified solution when iron, zinc, and manganese were present at 2 p. p. m. (2) The concentration of iron does not seem to be so important as the concentration of zinc. (3) The unpurified solution is decidedly more sensitive to increasing concentrations of zinc than is the purified solution. (It will be recalled from experiment 117 that the unpurified solution was also exceedingly

sensitive to copper application.) (4) A peculiar but unexplained response with iron application was observed in both the purified and unpurified solution. In the purified solution, 2, 5, and 10 p. p. m. gave about equal results but 20 p. p. m. gave a decided increase, and in the unpurified solution 20 p. p. m. gave an increase over 10 p. p. m., the most unfavorable combination. A somewhat similar relationship was noted with manganese in experiment 114 in the purified solution. (5) While the distinctions between 2 and 5 p. p. m. are not great and on the purified solution it has been almost impossible to decide whether 2 or 5 p. p. m. provided optimum growth conditions, it now seems that in the unpurified solution the best concentration for optimum conditions may definitely be stated as not more than a 0-2-2-2 concentration of copper, iron, manganese, and zinc; subsequent experiments have been based on this formula.

#### EFFECT OF IRON, MANGANESE, AND ZINC ON DIFFERENT ISOLATES

In some subsequent experiments on fungus nutrition it appeared that the presence of iron was not quite so important as the preceding experiments had indicated. This gave rise to speculation as to whether these results were caused by some uncontrolled factor, whether the fungus was losing some of its vegetative vigor, or whether different isolates might fail to respond uniformly to the treatments with the above elements that had been found necessary for optimum growth. Accordingly, the studies were extended to include additional isolates of the fungus collected during the summer of 1938 from various root rot-infested fields of central Texas. From the newly isolated cultures of *Phymatotrichum omnivorum* seven isolates were chosen for comparative studies with isolate 28, which had been isolated in 1937, and on which the preceding studies had been made.

Preliminary comparisons on solid media and on the unpurified nutrient solution indicated that the eight isolates differed in the amount of growth produced on the several substrates. For the more critical experiments to determine the response of isolates to the addition of certain elements, the number of isolates was reduced to four, isolate 28 being used as the standard and isolates 4, 5, and 7 being compared with it. Two experiments were conducted in the factorial design, the variable factors being isolates (4), treatments (5), and time of harvest (2 or 4). The treatments included were (0) no elements added to the unpurified solution, (A) zinc added, (B) iron and zinc added, (C) manganese and zinc added, and (D) iron, manganese, and zinc added, the elements, whether singly or in combination, being added at the rate of 2 p. p. m. each to the unpurified solution. In the first of these two experiments, designated as N-22, the mats were harvested at 14, 18, 22, and 26 days after inoculation, three replicates of each combination being used. The second experiment (N-26) was based upon harvests at 14 and 22 days, replicates four. The incubation was at 28° C. in both experiments. The response of the four isolates to the several treatments was much the same in both experiments, and an analysis of differences between the two experiments yielded no values which approached significance even at the 5-percent level, indicating that with the harvests at 14 and 22 days, common in both experiments, the two experiments might be considered as one and analyzed as such. The results of the 14- and 22-day harvests of the two experiments were, therefore, brought together in one analysis,

thus giving seven replicates. The summary of this analysis and the mean weights of the various combinations are presented in table 7. Since the analysis of differences at 14 and 22 days for experiments N-22 and N-26 suggested that the results might be analyzed together, it is assumed that conclusions may be drawn from either experiment as well as from the combined analysis. In N-22 there were four dates of harvest so that the growth curves obtained are more complete than could be obtained from N-26 or the combined analysis. Accordingly it seemed desirable to present these growth curves (fig. 3) and to base the interpretation and discussion of the results on the graph and the table of combined results.

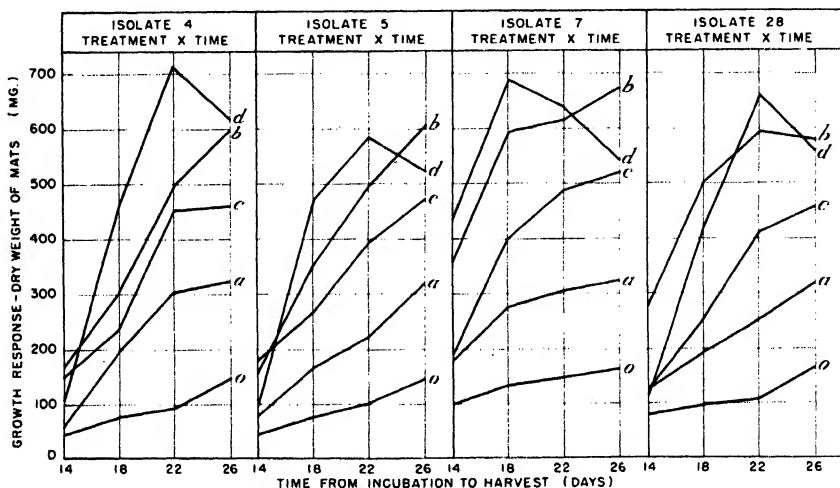


FIGURE 3.—Graphic representation of comparisons for experiment N-22. Dry weight of mats produced is used to measure the effect of several combinations of iron, manganese, and zinc at 2 p. m. on the growth of four isolates of *Phymatotrichum omnivorum* in unpurified solutions at 28° C. For each isolate treatment×time (or rate of growth) may be considered singly as a first-order interaction or the figure as a whole may be considered to represent the second-order interaction, isolate×treatment×time. 0, Check (all three elements omitted); a, zinc alone added; b, iron and zinc added; c, manganese and zinc added; d, iron, manganese, and zinc added.

*Isolates.*—Of the four isolates, No. 5 gave a comparatively low mat weight. This difference from the other three was highly significant. Isolates 4 and 28 gave similar mean mat weights, which differed significantly from the slow-growing isolate 5 and the rapid-growing isolate 7.

*Treatments.*—All treatments differed significantly from one another. The mean mat weight was greatest in treatment D (Fe, Mn, Zn), followed by treatment B (Fe, Zn), treatment C (Mn, Zn), treatment A (Zn), and treatment 0 (none added).

*Time×isolate.*—The four isolates responded in a similar manner to the differences in time of harvest when measured at 14 and 22 days only, and the mean square obtained in the combined analysis of variance scarcely exceeds that of error. However, this was not true when experiment N-22 was analyzed separately, and it is believed

that this would not have been the case had N-26 been harvested at four dates or times. Figure 3 shows that all treatments do not reach their peak at the same time, nor do all isolates respond equally well to treatments, particularly in the early stages of development. These differences may escape detection when the harvest is made at 14 and 22 days only.

TABLE 7.—Mean values and analysis of variance for reactions of four isolates of *Phymatotrichum omnivorum* to combinations of iron, manganese, and zinc

[Experiments N-22 and N-26 combined for yield at 14 and 22 days]

Variance	Mean mat weight		Analysis of variance			
	Mean	Difference required for significance (odds 99:1)	D. F.	Mean square <sup>1</sup>		
<b>Time of harvest</b>						
14 days	<i>Mg.</i> 170.09	<i>Mg.</i> 18.30	1	*4,112,316		
22 days	412.47					
<b>Isolates</b>						
No. 4	289.87	25.87	3	*95,392		
No. 5	247.13					
No. 7	337.51					
No. 28	290.62					
<b>Treatments</b>						
0	124.27	28.95	4	*917,603		
A	199.99					
B	302.52					
C	311.79					
D	427.83					
Time×isolates			3	4,981		
<b>Mean mat weight with isolate—</b>						
	No. 4	No. 5	No. 7	No. 28		
<b>Time of harvest:</b>						
14 days	<i>Mg.</i> 158.07	<i>Mg.</i> 131.19	<i>Mg.</i> 224.43	<i>Mg.</i> 166.68		
22 days	421.67	363.07	450.59	414.55		
Time×treatments			4	*257,713		
<b>Mean mat weight with treatment—</b>						
	0	A	B	C	D	
<b>Time of harvest:</b>						
14 days	<i>Mg.</i> 88.56	<i>Mg.</i> 126.66	<i>Mg.</i> 215.37	<i>Mg.</i> 188.55	<i>Mg.</i> 231.31	
22 days	159.98	273.31	509.67	435.04	624.35	
<b>Isolates×treatments</b>						
Isolate—						
No. 4	117.01	214.54	341.01	319.90	456.90	
No. 5	98.22	163.19	317.35	284.49	372.41	
No. 7	144.35	227.98	490.26	341.21	483.72	
No. 28	137.51	194.24	421.46	301.58	398.29	
<b>Time×isolates×treatments</b>						
14 days, isolate—						
No. 4	79.57	123.01	175.99	187.73	224.06	
No. 5	68.33	86.27	149.81	176.69	174.87	
No. 7	111.93	164.83	290.27	215.74	339.36	
No. 28	94.43	132.53	245.41	174.04	186.97	
22 days, isolate—						
No. 4	154.46	306.06	506.03	452.07	689.74	
No. 5	128.11	240.11	484.89	392.29	569.96	
No. 7	176.77	291.13	690.26	466.69	628.09	
No. 28	180.59	255.94	597.51	429.11	609.61	
Error						
					249	3,468
Total					279	36,486

<sup>1</sup> Asterisk (\*) indicates that contributor is highly significant (odds 99:1).



*Time*×*treatment*.—It will be seen that with treatment 0 the mean mat weight was not doubled during the period from 14 to 22 days, whereas with treatments B and D the weights had increased more than two and one-half times during the same period and high significance was obtained.

*Isolate*×*treatment*.—This interaction was highly significant, owing largely to the fact that strain 4 responded unusually well to treatment D (Fe, Mn, Zn), whereas, with isolates 7 and 28 at 22 days, treatments B and D were not very different from each other. Treatment C is only slightly inferior to treatment B for isolate 4, but in relation to the better treatments it gives comparatively poor results for isolates 7 and 28. It is interesting to note from figure 3 that for the poorer treatments, C, A, and 0, the final mat weights at 22 days from each such treatment are not actually very different for the four isolates.

*Time*×*isolate*×*treatment*.—This interaction does not approach significance in the combined analysis. However, in the analysis of N-22 based upon four times of harvest, a significant value was obtained. The explanation of this has been pointed out in the discussion of the interaction *time*×*isolate*.

In conclusion, the most important features of the foregoing test may be summarized with the following statements. On the basis of mean mat weights attained with different treatments, isolates 4 and 28 are classed together, intermediate in position between isolate 7, or a significantly higher group, and isolate 5, of a lower group. Although, isolates 7 and 28 are in different subgroups on the basis of mean weights it has been pointed out that these two isolates react similarly in their response to the various treatments. It is of possible significance that isolates 7 and 28 were originally obtained from the same location in a single root rot spot in an otherwise root rot-free cotton field. The collections were made in 2 successive years. Their differential response to treatments suggests their identity, despite the fact that their growth rate was different. The latter may possibly be explained by the fact that the slower growing isolate had been carried in culture for a year longer than the faster growing one. It has been commonly observed with other fungi that differences may be detected in vegetative vigor following continued growth on artificial media. Consequently it was not so surprising to find that the various isolates differed in vegetative vigor or growth rate as it was to discover that isolates of *Phymatotrichum omnivorum* responded differentially to the various combinations of iron, manganese, and zinc.

#### FURTHER EXPERIMENTS WITH OTHER TRACE ELEMENTS

Earlier experiments have failed to demonstrate beneficial results from the addition of certain of the trace elements (aluminum, boron, etc.) to the purified solution when added to yield concentrations ranging from 1 to 10 p. p. m. Although these elements alone failed to give increases in mat weight, it was considered possible that if they were used in combination with iron, manganese, and zinc a further response in mat weight might be obtained. Accordingly, to the purified solution plus iron, manganese, and zinc (at 2 p. p. m. each) the elements were added singly to portions of the solution to give concentrations of 2, 4, or 10 p. p. m. The various treatments were set up in quadruplicate and were harvested after 21 days' incubation at 28° C. The results of this experiment are summarized in table 8.

With regard to the differences between the elements, it may be stated that a preliminary analysis of variance was made for each rate in order to establish the differential response of the fungus to the 11 elements under consideration when iron, manganese, and zinc were present. It was found that more than 58 mg. but less than 59 mg. was required for highly significant differences between the means of any 2 elements at both 2 and 4 p. p. m. None of the elements was significantly better or poorer than the check at 2 p. p. m., nor at 4 p. p. m. with the exception of nickel, which significantly depressed growth, and aluminum, which caused an increase over the check that approached significance. However, at both 2 and 4 p. p. m. certain of the better treatments could be differentiated from the poorer. It did not seem worth while to present the details of the separate analyses since the combined analysis at the 3 rates (table 9) seemed to bring out all the essential facts mentioned above, plus the additional information concerning rates and interactions of rate  $\times$  element.

TABLE 8.—*Response of Phymatotrichum omnivorum to the addition of various elements to a purified solution containing Fe, Mn, and Zn, incubated at 28° C. for 21 days (experiment 120)*

Element <sup>1</sup>	Mean mat weight at—			Mean <sup>2</sup>
	2 p. p. m.	4 p. p. m.	10 p. p. m.	
Aluminum	Mg. 605.2	Mg. 667.0	Mg. 633.7	Mg. 635.32
Fluorine	616.0	642.2	638.4	632.24
Boron	611.8	634.2	601.0	615.63
Mercury	617.7	599.9	618.5	612.04
Silicon	589.4	614.4	597.4	600.42
Iodine	602.4	616.9	560.8	593.34
Cadmium	570.9	591.7	589.1	583.89
Molybdenum	553.7	550.6	578.1	560.79
Cobalt	603.9	570.4	43.3	405.86
Nickel	575.5	535.9	14.9	375.43
Mean <sup>2</sup>	594.65	602.34	487.50	

<sup>1</sup> The following results, which were not included in table 8 nor in the analysis given in table 9, were secured with the elements or the checks indicated: Lithium at 4 p. p. m. gave 583.5 mg.; 10 p. p. m., 572.2 mg. Check A, with Fe, Mn, and Zn at 2 p. p. m., but other trace elements absent, gave 608.9 mg. Check B, with Fe, Mn, and Zn at 2 p. p. m. and also lithium and all of the 10 trace elements listed in the above table at 2 p. p. m., gave 577.3 mg.

<sup>2</sup> Difference required for significance (odds 99:1): Elements=34.51 mg.; Rates=18.90 mg.

For the analysis only 10 of the 11 elements were considered, since the lithium series was not complete for all 3 rates. A subsequent test with lithium showed no material difference for rates at 2, 4, and 10 p. p. m. Comparisons were made of the 10 elements at the 3 rates, and it will be observed that there was little difference between the 2 lowest rates, the mean for 2 p. p. m. being 594.65 mg. and for 4 p. p. m. 602.34 mg. The addition of cobalt or nickel at 10 p. p. m. resulted in almost complete inhibition of growth, whereas the other elements behaved very much as at 2 and 4 p. p. m. The depression of growth with the addition of cobalt or nickel at the rate of 10 p. p. m. resulted in a highly significant difference in the comparison of the mean of the rates of 2 plus 4 p. p. m. with 10 p. p. m.

In the analysis, which includes all three rates, it is also evident that no element is distinctly superior to the check, but certain group

responses can be identified. In order to emphasize this grouping, the 9 degrees of freedom for elements were broken down in the manner explained in footnote 2 of table 9.

TABLE 9.—*Analysis of variance for experiment 120*

Variance	D. F.	Mean square	F		
			Found	Required	
				1 percent	5 percent
Total	119	22,053.9345			
Rates: <sup>1</sup>					
R <sub>1</sub>	1	1,183.4911	1.15	6.92	3.95
R <sub>2</sub>	1	328,552.6079	318.46	6.92	3.95
Elements: <sup>2</sup>					
E <sub>1</sub>	1	5,554.0838	5.38	6.92	3.95
E <sub>2</sub>	1	3,201.6600	3.10	6.92	3.95
E <sub>3</sub>	1	300.3538			
E <sub>4</sub>	1	77.4004			
E <sub>5</sub>	1	56.7338			
E <sub>6</sub>	1	7,225.0669	7.00	6.92	3.95
E <sub>7</sub>	1	4,772.0408	4.63	6.92	3.95
E <sub>8</sub>	1	36,875.4401	35.74	6.92	3.95
E <sub>9</sub>	1	875,700.2175	848.80	6.92	3.95
Rates×Elements	18	70,448.1485	68.28	2.20	1.75
Error	90	1,031.6039			

<sup>1</sup> Comparisons between rates:

R<sub>1</sub>=2 p. p. m. compared with 4 p. p. m.

R<sub>2</sub>=Mean of rates 2 and 4 p. p. m. compared with 10 p. p. m.

<sup>2</sup> Comparisons between elements:

E<sub>1</sub>=Co compared with Ni.

E<sub>2</sub>=Cd compared with Mo.

E<sub>3</sub>=Si compared with I.

E<sub>4</sub>=B compared with Hg.

E<sub>5</sub>=Al compared with F.

E<sub>6</sub>=Mean effect of Si and I compared with mean effect of Cd and Mo.

E<sub>7</sub>=Mean effect of Al and F compared with mean effect of B and Hg.

E<sub>8</sub>=Mean effect of Al, F, B, and Hg compared with mean effect of Si, I, Cd, and Mo.

E<sub>9</sub>=Mean effect of Al, F, B, Hg, Si, I, Cd, and Mo compared with mean effect of Co and Ni.

It will be noted that cobalt and nickel (comparison E<sub>1</sub>) both inhibit growth almost completely at 10 p. p. m., nickel slightly more than cobalt. However, the difference in the extent to which growth was depressed by these elements does not reach significance at the 1-percent requirement. As is shown by the mean square values obtained for E<sub>2</sub>, E<sub>3</sub>, E<sub>4</sub>, and E<sub>5</sub>, for comparison of cadmium with molybdenum, silicon with iodine, boron with mercury, and aluminum with fluorine, respectively, the individual differences are insignificant. E<sub>6</sub>, showing the mean effect of silicon and iodine as compared with cadmium and molybdenum, reaches high significance, while E<sub>7</sub>, comparing the mean effect of aluminum and fluorine with boron and mercury, exceeds the 5-percent but does not reach the 1-percent requirement. In E<sub>8</sub> the four elements aluminum, fluorine, boron, and mercury, which consistently gave higher values than the check series (608.9 mg.), are compared with the next four elements, silicon, iodine, cadmium, and molybdenum. A highly significant *F* value is obtained for this comparison. The comparison of the mean effect of the eight elements considered in E<sub>8</sub> with the mean of cobalt and nickel yields a high *F* value, as might be expected. For the interaction of rates×elements, a high *F* value is obtained, and a partial explanation of this is afforded by the fact that eight of the elements maintain a moderately constant mat weight at all rates as contrasted with the marked depression of mat weights with cobalt and nickel with increases in

concentration. Although the nutrient solution was highly favorable for growth of the organism, cobalt and nickel showed the same high degree of toxicity at 10 p. p. m. as was shown in earlier experiments on the purified solution to which iron, manganese, and zinc had not been added following purification. However, cobalt does not show in this experiment the severely depressing effect at 4 p. p. m. that resulted in the earlier experiments.

A similar experiment was conducted on the unpurified solution with iron, manganese, and zinc, added at the rate of 2 p. p. m. each, and with the other elements added singly at the same rate. The mean weights, based upon five replicates of the various treatments, were as follows:

Element added:	Mg.	Element added:	Mg.
B.....	732.4	Mo.....	618.9
Al.....	729.9	F.....	610.1
Cd.....	718.3	Hg.....	587.7
Si.....	704.8	Li.....	568.5
Control.....	688.1	Co.....	553.3
Ni.....	674.2	I.....	526.6

The difference required between means for significance at odds of 99:1 was 138.6 mg. No significant increases in mat weight over the control were obtained through the addition of these elements, but it is of interest to note that boron and aluminum rank first and second, while in the preceding experiment they ranked third and first, respectively. Decreases in mat weight were obtained with the addition of a number of the elements, but only with iodine was the difference highly significant, although cobalt closely approached the requirement.

On the basis of these experiments it may be concluded that the addition of the trace elements aluminum, boron, etc., to purified or unpurified solutions containing iron, manganese, and zinc fails to give significant increases in mat weight over that obtained with the iron-manganese-zinc combination alone. These results suggest that the trace elements in the former group are not essential to *Phymatotrichum omnivorum* or that they are furnished in sufficient quantities as impurities, either in the solution or in the inoculum piece, to insure good growth of the fungus.

#### SUMMARY AND CONCLUSIONS

In an effort to develop the most satisfactory medium possible for certain fundamental studies with the root rot organism (*Phymatotrichum omnivorum*), several experiments were made to find what elements are needed for optimum growth and the proper proportion of each.

Of the four trace elements copper, iron, manganese, and zinc, which were studied intensively in these experiments, it appeared that at least three are essential for the optimum growth of *Phymatotrichum omnivorum*. Preliminary studies revealed the surprising fact that a very much higher concentration of these elements was required than had been found to be the case for several of the organisms studied by other workers.

Other trace elements—aluminum, boron, cadmium, cobalt, fluorine, iodine, lithium, mercury, molybdenum, nickel, and silicon—were found to be nonessential for optimum growth under the conditions employed in these studies. This was true whether iron, manganese,

and zinc were present or absent. It may be noted in passing that aluminum and boron were fairly consistent in giving a slight increase in growth over the control. Certain of the elements, particularly nickel and cobalt, were highly toxic at more than 4 p. p. m., in the purified solution, and some depression was caused at 4 p. p. m., particularly with nickel.

The use of the factorial design provided a convenient and highly effective method for studying the reaction of the organism to these elements, particularly in evaluating interactions.

In the standard solution, the addition of copper, iron, manganese, and zinc did not produce as good growth as could be obtained on the purified solutions to which these elements were added. Furthermore, in the preliminary studies with unpurified solution, it was believed that the amounts of impurities contained might be a serious factor in evaluating the response of the organism through additions of the elements.

The results of three factorial experiments for determining the effect of the presence or absence of copper, iron, manganese, and zinc in purified solutions are discussed. It was found that copper was somewhat valuable at 2 p. p. m. and unimportant at 5 p. p. m., and that it caused a slight depression at 10 p. p. m. With iron, manganese, and zinc present, very marked increases in growth occurred, much more so than could be accounted for by summation of their effects when used singly, thereby indicating very important interactions. Interaction was greatest for combinations of iron and zinc and manganese and zinc.

In subsequent factorial experiments it developed that the unpurified solution gave results equal or superior to those obtained with the purified solution, provided copper was entirely omitted and iron, manganese, and zinc were employed in about the same ratios. It seemed to matter little whether these rates were 2-2-2 or 5-5-5 p. p. m., although later experiments indicated that the 2-2-2 p. p. m. rate might be considered as optimum for growth of the organism in the unpurified solution. The unpurified solution was extremely sensitive to the addition of copper and was very much more sensitive than the purified solution to increasing concentrations of iron and zinc, particularly the latter.

In further experiments in which iron and zinc were varied at four different levels each, while manganese was held constant at 2 p. p. m., the importance of these relative ratios became further evident and the harmful effects resulting from an unbalanced condition were stressed, particularly in the inhibiting effects of increased concentrations of zinc in relation to the iron concentration. One unexplained anomaly was the depressing effect of iron at 10 p. p. m. in the unpurified solution, an effect which largely disappeared at higher concentrations.

In an attempt to determine whether continued growth on artificial media had resulted in the deterioration of the various isolates of the organism, and whether all isolates would utilize equally well the elements that had been shown to be necessary for optimum growth, three new isolates of *Phymatotrichum omnivorum* were compared with each other and with the standard strain, which had been in culture for over a year. The results showed that these isolates could be distinguished by their rate of growth and by their ability to utilize different combinations of iron, manganese, and zinc.

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# THEORETICAL ANALYSIS OF SMOKE - COLUMN VISIBILITY<sup>1</sup>

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## INTRODUCTION

As part of a general forest-fire-research program in recent years, considerable inquiry has been made into the visibility of smoke from forest fires in an attempt to answer the questions: How far can a lookout see a smoke? What are the factors upon which this visual range depends? In what way and to what extent do these factors exert their influence? In the answer to these questions are embodied principles upon which a forest-fire-detection system should be based and upon which practical administrative action should be taken in establishing lookouts and in posting supplementary observers in time of emergency.

Byram (4) <sup>3</sup> has observed variations in visibility distance with haze of a small smoke against a black background and has reasoned that this visibility distance would be modified by variations in background brightness, position of the sun, illumination, and magnification or increase in size of the smoke body. Buck and Fons (2) made a laboratory study of the effect of direction of illumination and of suspended concentration upon smoke brightness and Buck (1), by analysis of forest-fire discovery-time data, has stressed the importance of the angle between sun, smoke, and observer as influencing visibility. There is need for a more complete theoretical analysis of this problem, starting from defined fundamental properties, with a clear understanding of the assumptions and approximations involved. Such an analysis should serve as a basis for the development of ideas and for the improvement and standardization of techniques.

With this aim in mind this paper was prepared, not in any attempt to solve the whole problem of smoke visibility, but rather to treat of the theoretical aspects and to develop equations for visual smoke range to the point where experimental observations are required to evaluate constants and to determine unknown functions. The equations, if acceptable, should serve as a working hypothesis and guide in the experimentation needed for complete or practical solution of the problem.

## GLOSSARY OF SYMBOLS

- $\phi$  = Light flux entering the observer's eye.
- $d\phi$  = Flux reaching the eye from an infinitesimal element of surface  $da$ .
- $\phi_1$  = Flux reaching the eye from the haze between the observer and the smoke.
- $\phi_2$  = Flux reaching the eye from the smoke.

<sup>1</sup> Received for publication August 7, 1940.

<sup>2</sup> Maintained in cooperation with the University of California, Berkeley, Calif.

<sup>3</sup> Numbers in italics refer to Literature Cited, p. 178.



- $\phi_3$  = Flux reaching the eye from the haze between the smoke and the background.
- $\phi_4$  = Flux reaching the eye from the background in the direction adjacent to the smoke column.
- $\phi_5$  = Flux reaching the eye from the background in the direction of the smoke column.
- $\phi_6$  = Flux reaching the eye from the haze between the observer and the background in the direction adjacent to the smoke column.
- $\phi_s$  = Flux reaching the eye from the direction of the smoke column.
- $\phi_b$  = Flux reaching the eye from the direction of the background adjacent to the smoke column.
- $a$  = Area in the field of view perpendicular to the line of sight.
- $da$  = An infinitesimal element of the surface  $a$ .
- $A$  = Apparent reflectance (9) of an element of the background adjacent to the smoke column; also used with subscripts  $s$ ,  $h$ , and  $\epsilon$  to refer to the sunlight, skylight, and earthlight respectively. Apparent luminous reflectance is the ratio of light reflected to light incident on a perfectly diffusing surface of such reflectance that it has the same brightness, viewed at close range, as the sample surface similarly illuminated and viewed. It is a function of the spectral and angular distribution of the incident light and of the direction of view. If the surface were perfectly diffusing,  $A$  might be regarded as the intrinsic brightness of the background and as equal to the diffuse reflection coefficient.
- $B$  = Brightness, in general.
- $B_b$  = Apparent brightness of the background, viewed at a distance.
- $B'_b$  = Apparent brightness of the background, viewed at close range.
- $B_{sb}$  = Apparent brightness of the smoke, viewed at a distance against a terrestrial background.
- $B_{sh}$  = Apparent brightness of the smoke, viewed at a distance against the horizon sky.
- $B'_{sb}$  = Apparent brightness of the smoke, viewed at close range against a terrestrial background.
- $B'_{sh}$  = Apparent brightness of the smoke, viewed at close range against the horizon sky.
- $B_h$  = Apparent brightness of the horizon sky adjacent to the smoke column or to the background.
- $l$  = Distance from the observer to the background.
- $l'$  = Distance from the observer to the smoke.
- $l^*$  = That particular value of  $l'$  at which the smoke can barely be distinguished.
- $v$  = The fractional difference between two brightnesses barely distinguishable by the normal human eye.
- $x$  = General distance along the eye-to-smoke axis.
- $Z_1$  = The angular scattering function for an average particle of hazy atmosphere.  $Z_1$  is a function of the spacial distribution of the light incident upon the particle as well as of the distribution of the scattered light.
- $Z_2$  = The angular scattering function for an average particle in a volume of smoke.  $Z_2$  is a function both of the spacial distribution of the light incident upon the particle and of the scattered distribution.
- $\Delta\omega$  = The solid angle subtended by the pupil of the observer's eye at the element  $da$ .
- $\rho$  = The coefficient of light attenuation by the hazy atmosphere, defined by  $dI = -\rho I dx$  in which  $dI$  symbolizes the change in transmitted intensity due to scattering and absorption in an incident beam of intensity  $I$  in the distance  $dx$ .  $(1-\rho)$  = transmission coefficient.  $\rho$  = scattering + absorption coefficients.  $e^{-\rho x}$  = the fraction of the incident light transmitted through a distance  $x$  of hazy atmosphere.
- $\alpha$  = The scattering coefficient of the hazy atmosphere, defined by  $dI = -\alpha I dx$ , in which  $dI$  represents the change in transmitted intensity due to scattering alone.
- $\sigma$  = The coefficient of light attenuation by the smoke (analogous to  $\rho$  for the haze).
- $\gamma$  = The scattering coefficient of the smoke (analogous to  $\alpha$ ).
- $\alpha_1$  = The scattering power of a single average particle of the hazy atmosphere.
- $\gamma_1$  = The scattering power of a single average particle of the volume of smoke.
- $n$  = Number of scattering particles.
- $c$  = Volume concentration of scattering particles.

- $\theta$  = The angle of deviation for sunlight of the scattered ray from the path of the incident ray.  
 $\psi$  = The angle of inclination of the earth-surface element from the horizontal toward the observer.  
 $t$  = Effective thickness or diameter of the smoke column.  
 $\bar{d}$  = Mean effective length of the path of the illuminating light from the outer surface into the body of the smoke column.  
 $I$  = Intensity of incident illumination; also used with subscripts  $s$ ,  $h$ , and  $e$  to refer to the sunlight, skylight, and earthlight, respectively.  
 $w$  = Wind intensity.  
 $m$  = Magnification.

### GENERAL THEORY

A smoke is visible largely because of a contrast in brightness with the adjacent background.<sup>4</sup> This contrast may be expressed in terms of the "contrast ratio" defined as follows:

$$\text{Contrast ratio} = \frac{\text{Apparent brightness of the background}}{\text{Apparent brightness of the smoke column}}$$

Ordinarily, the smoke will appear brighter than its background and the contrast ratio will vary between zero and unity. Zero would mean extreme contrast and maximum visibility. At a ratio of 1, there would be no contrast and the smoke could not be discerned. A sooty smoke or a dense smoke illuminated from behind may appear darker than its background, especially if the latter be the horizon sky, and the contrast ratio as defined would then be greater than unity.

An expression is required for the contrast ratio between a smoke column and its background in terms of defined and measurable quantities.

Consider an eye observing a distant smoke against a solid background as represented in figure 1. The luminous flux to enter the pupil of the eye from the two directions shown will be  $\phi_s$ <sup>5</sup> and  $\phi_b$ . The former will consist of the sum of  $\phi_1$ ,  $\phi_2$ ,  $\phi_3$ , and  $\phi_5$ . The latter will equal  $\phi_4 + \phi_6$ . Our procedure will be to express each one of the quantities  $\phi_1$ ,  $\phi_2$ ,  $\phi_3$ ,  $\phi_4$ ,  $\phi_5$ , and  $\phi_6$  separately in order to determine  $\phi_s$  and  $\phi_b$ . Then, by changing  $\phi$ , flux, to  $B$ , brightness, we will have  $B_{sb}$  and  $B_b$ , the ratio of which will be the contrast ratio that we seek.

It is assumed that in all cases essentially white light or light of constant hue is involved.

### FLUX FROM THE BACKGROUND $\phi_4$ AND $\phi_5$

Imagine an observer viewing a distant background. His eye will focus upon only one surface element at a time, and no more than the one need be considered, since any other would be brought to a focus on the retina at a different point, and it is assumed that the immediate background is of uniform brightness. Let the projection of the element perpendicular to the line of sight be  $da$  and the amount of light flux to reach the eye from the element be  $d\phi_4$ . Consider the background surface inclined toward the observer at an angle  $\psi$  from the horizontal, and assume the normal to the plane of the element to

<sup>4</sup> There is also some contrast in hue, but it is very slight for smokes at such a distance that they are barely distinguishable. Accordingly, in this study hue contrast is considered negligible. Movement plays its part in enabling the observer to conclude that some foreign aspect of the landscape is or is not smoke, but not in rendering that object initially visible.

<sup>5</sup> All terms are defined in the Glossary of Symbols, pp. 161-163.

lie in the plane through the sun and the line of sight. Under these conditions, the point observed would be the high light if the background were glossy; hence a natural background would be of maximum brightness and a smoke viewed against it would be least apparent.

Consider for the moment sun illumination only. The background

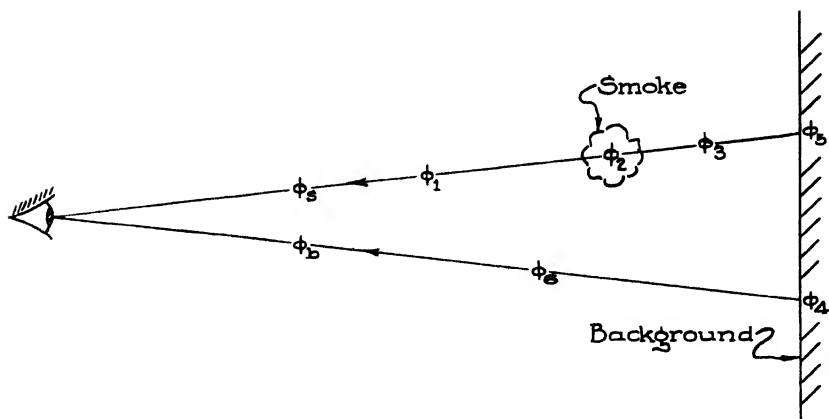


FIGURE 1.—Schematic drawing showing the six sources of the light flux that reaches an observer viewing a body of smoke against a distant background.

will be illuminated by sunlight at an angle  $\theta$ , as illustrated in figure 2. From the projected element of surface  $da$  a certain amount of sunlight will be reflected through the solid angle  $\Delta\omega$  and will enter the pupil. If  $I_s$  be the full intensity of the sunlight, the illumination in terms of flux per unit area on the inclined surface is  $I_s \sin(\theta - \psi)$ . The

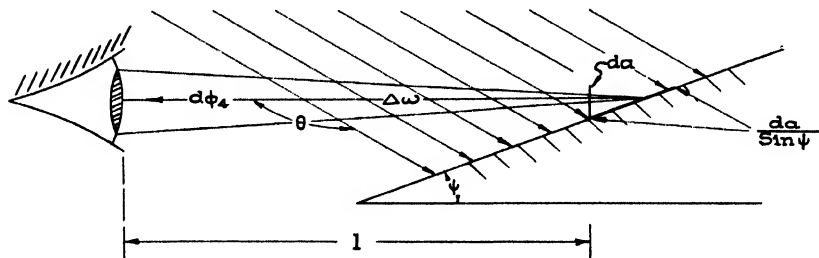


FIGURE 2.—Schematic drawing to show flux reaching the eye of an observer viewing a distant background illuminated by sunlight.

apparent reflected sunlight flux from unit surface is  $I_s A_s \sin(\theta - \psi)$ , and from the element of surface is  $I_s A_s \sin(\theta - \psi) da$ .  $\frac{I_s}{\pi} A_s \sin(\theta - \psi) da \Delta\omega$  is the portion of this reflected flux directed towards the eye and, since this flux will be diminished by attenuation in transit, the sunlight flux to enter the pupil will be

$$\frac{I_s}{\pi} A_s \sin(\theta - \psi) da \Delta\omega e^{-\rho t}$$

in which  $e^{-\rho l}$  is the fraction of the light transmitted through a distance  $l$  of hazy atmosphere of attenuation coefficient  $\rho$ .

The element of surface is illuminated not only by sunlight but also by skylight and earthlight. These illuminations depend on  $\psi$  in an unknown manner, for which the general symbols  $f_h(\psi)$  and  $f_e(\psi)$  will be used to denote the surface integrals between the normal to the earth-surface element and the horizon over the sky and the earth, respectively. Thus the skylight flux to reach the eye from the surface element will be  $I_h A_h f_h(\psi) da \Delta\omega e^{-\rho l}$  and the earthlight flux will be  $I_e A_e f_e(\psi) da \Delta\omega e^{-\rho l}$ .

The total flux,  $d\phi_4$ , to reach the eye from the earth-surface element will be the sum of the above three quantities.

$$d\phi_4 = \frac{I_s}{\pi} A_s \sin(\theta - \psi) da \Delta\omega e^{-\rho l} + I_h A_h f_h(\psi) da \Delta\omega e^{-\rho l} + I_e A_e f_e(\psi) da \Delta\omega e^{-\rho l} \quad (1)$$

The first (sunlight) term of the right-hand side of equation (1) is ordinarily the predominating one, but it would be absent when the sky is completely overcast or when the background is in shadow. The sunlight term has no real physical significance when  $(\theta - \psi)$  is negative. When  $\theta = \psi$ , there will be grazing incidence and zero illumination by the sunlight. The third (earthlight) term is probably negligible except when the earth is blanketed with snow.

For the sake of simplicity, equation (1) may be put in the form

$$d\phi_4 = I A f(\theta, \psi) da \Delta\omega e^{-\rho l} \quad (2)$$

in which  $I$  is the intensity of illumination from all sources and  $A$  is the concomitant apparent luminous reflectance.

Should the light from the background pass through a volume of smoke of attenuation coefficient  $\sigma$  and thickness  $t$ , the flux to reach the eye would be  $d\phi_5$ .

$$d\phi_5 = d\phi_4 e^{-\sigma t} = I A f(\theta, \psi) da \Delta\omega e^{-\rho l} e^{-\sigma t} \quad (3)$$

For very dark backgrounds and for very dense smokes  $d\phi_5$  becomes negligible.

#### FLUX FROM THE HAZE BETWEEN OBSERVER AND BACKGROUND, $\phi_6$

To obtain  $d\phi_6$ , let  $da$  now represent the surface of an infinitesimal cylinder of haze of length  $dx$  (fig. 3). Imagine a myriad of very small particles in this cylindrical element of volume, each scattering independently of the others, so that the scattered intensity is proportional to the number of particles.

The intensity of the light scattered in any particular direction by the infinitesimal volume of hazy air will be proportional to the incident intensity  $I$ , to the number  $dn$  of scattering centers in the volume and to the ability  $\alpha_1$  of each particle to scatter light. The proportionality constant  $Z_1$  involves the angular distribution of the incident light as well as the angular distribution of the scattered light.  $\alpha_1$  is characteristic of the air molecules and haze particles and represents

a weighted mean for the diversity of kind, size, and shape of scattering particles present. Hence the total light flux scattered by the elemental volume as in figure 3 will be proportional to  $I \, dn \, \alpha_1$ , or  $I \alpha_1 c \, da \, dx$ , or  $I \propto da \, dx$  in which  $\alpha = c \, \alpha_1$ . The flux scattered in the direction of the eye will be  $Z_1 I \propto da \, dx \, \Delta \omega$  and, of this,  $Z_1 I \propto da \, dx \, \Delta \omega \, e^{-\rho x}$  will reach the eye and enter the pupil. This quantity summed up for every similar little element of haze between the observer and the background will be  $d\phi_0$ .

$$d\phi_0 = Z_1 I \propto da \, \Delta \omega \int_0^l e^{-\rho x} dx = Z_1 I \propto da \, \Delta \omega \frac{\alpha}{\rho} (1 - e^{-\rho l}) \quad (4)$$

In this integration a uniform haze is assumed to exist between the eye and the background, and the line of sight is assumed to be uniformly illuminated by sunlight, skylight, and earthlight. This latter assumption would be severely violated by an unusually bright or

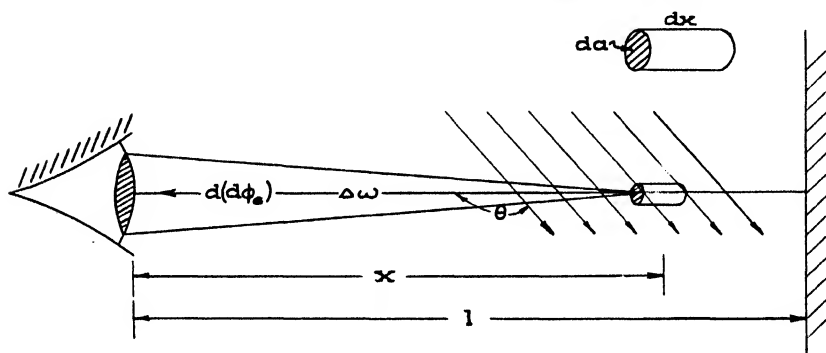


FIGURE 3. Schematic drawing to show flux reaching the eye of an observer viewing an element of haze illuminated by sunlight.

dark cloud on that part of the horizon which is near the smoke column, or by a shadow cast by a heavy cloud over a part of the line of sight.

#### FLUX FROM THE HAZE BETWEEN OBSERVER AND SMOKE, $\phi_1$

In analogy to equation (4), we may write at once

$$d\phi_1 = Z_1 I \propto da \, \Delta \omega \frac{\alpha}{\rho} (1 - e^{-\rho l}) \quad (5)$$

#### FLUX FROM THE HAZE BETWEEN SMOKE AND BACKGROUND, $\phi_2$

In analogy to equation (4) except that in this case the haze light passes through the smoke, which transmits only the fraction  $e^{-\sigma t}$ , we have

$$d\phi_2 = Z_1 I \propto da \, \Delta \omega \alpha e^{-\sigma t} \int_0^l e^{-\rho x} dx$$

$$d\phi_2 = Z_1 I \propto da \, \Delta \omega \frac{\alpha}{\rho} (e^{-\rho l} - e^{-\rho l}) e^{-\sigma t} \quad (6)$$

For dense smoke and smokes close to the background  $d\phi_2$  is negligible.

FLUX FROM THE SMOKE COLUMN,  $\phi_2$ 

Consider, now, only light scattered from the smoke column. If the angular scattering function for a smoke particle<sup>6</sup> be  $Z_2$  and if  $I$  be the incident intensity from all sources, then  $Z_2 I \, dn \, \gamma_1$  is the scattered intensity in a particular direction, such as the direction of the observer, i. e., the flux sent through unit solid angle from  $dn$  particles of the matter comprising a volume of the smoke, each on the average with ability to scatter flux denoted by  $\gamma_1$ . Like  $Z_1$ ,  $Z_2$  is a function of the incident distribution as well as of the scattered distribution.

Considering the  $dn$  particles to be in a cylindrical volume of smoke of  $da$  face area and  $dx$  length, the intensity of the illumination on this elemental volume will be  $I e^{-\sigma \bar{d}}$ , and the total scattered flux will be proportional to  $I e^{-\sigma \bar{d}} \gamma_1 \, dn$ , or  $I e^{-\sigma \bar{d}} \gamma_1 \, c \, da \, dx$ , or  $I e^{-\sigma \bar{d}} \gamma \, da \, dx$  in which  $c$  = the concentration of scattering particles in the smoke, and  $\gamma = c \gamma_1$ .

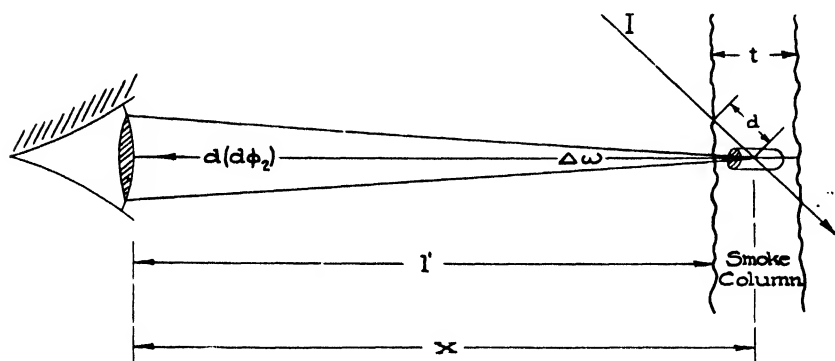


FIGURE 4.—Schematic drawing to show flux reaching the eye of an observer viewing an element of smoke illuminated by sunlight.

The flux scattered in the direction of the eye subtending the angle  $\Delta\omega$  will be  $Z_2 I \gamma \, da \, dx \, \Delta\omega e^{-\sigma \bar{d}}$ . Of this flux scattered from the elemental volume of smoke toward the eye, the flux to reach the front edge of the smoke column will be  $Z_2 I \gamma \, da \, dx \, \Delta\omega e^{-\sigma(x-l')} e^{-\sigma \bar{d}}$ . This is illustrated in figure 4. The flux scattered from the volume  $t \, da$  toward the eye is

$$Z_2 I \gamma \, da \, \Delta\omega \, e^{-\sigma \bar{d}} \int_{l'}^{l'+t} e^{-\sigma(x-l')} \, dx \text{ or } Z_2 I \frac{\gamma}{\sigma} \, da \, \Delta\omega (1 - e^{-\sigma t}) e^{-\sigma \bar{d}} \quad (7)$$

In this integration it is assumed that  $\gamma$  and  $\sigma$  are not functions of  $x$ ; that is, that the smoke concentration is uniform in all parts.  $\bar{d}$ , by definition, is not a function of  $x$ .

The flux to reach the eye from the volume  $t \, da$  is  $d\phi_2$ .

$$d\phi_2 = Z_2 I \frac{\gamma}{\sigma} \, da \, \Delta\omega (1 - e^{-\sigma t}) e^{-\sigma \bar{d}} e^{-\sigma l'} \quad (8)$$

<sup>6</sup> Since the particles which scatter light are not only the smoke ash suspensoid but also droplets of water vapor and molecules of gas and air, all both charged and uncharged,  $Z_2$  must include the effective scattered light distribution for the mixture as encountered in practice.

## APPARENT BRIGHTNESS OF A BACKGROUND

The luminous flux which reaches the observer's eye from the direction of the background is  $\phi_b$ . It comes not only from the background but also from the intervening haze, as shown in figure 1. Whence,

$$d\phi_b = d\phi_4 + d\phi_6$$

By equations (2) and (4)

$$d\phi_b = I Af(\theta, \psi) da \Delta\omega e^{-\rho l} + Z_1 I da \Delta\omega \frac{\alpha}{\rho} (1 - e^{-\rho l}) \quad (9)$$

Since brightness is defined as flux per unit solid angle per unit area,

$$B = \frac{d\phi/\Delta\omega}{da} \quad (10)$$

with which equation (9) may be converted to

$$B_b = I Af(\theta, \psi) e^{-\rho l} + Z_1 I \frac{\alpha}{\rho} (1 - e^{-\rho l}) \quad (11)$$

## APPARENT BRIGHTNESS OF A SMOKE COLUMN

The luminous flux which reaches the observer's eye from the direction of the smoke column is  $\phi_s$ . It arises from four sources as shown in figure 1:

$$d\phi_s = d\phi_1 + d\phi_2 + d\phi_3 + d\phi_5$$

By equations (5), (8), (6), (3), and (10),

$$\begin{aligned} B_{sb} = & Z_1 I \frac{\alpha}{\rho} (1 - e^{-\rho \gamma}) + Z_2 I \frac{\gamma}{\sigma} (1 - e^{-\sigma l}) e^{-\sigma \bar{d}} e^{-\rho \gamma} \\ & + Z_1 I \frac{\alpha}{\rho} (e^{-\rho l'} - e^{-\rho l}) e^{-\sigma l} + I Af(\theta, \psi) e^{-\rho l} e^{-\sigma l} \end{aligned} \quad (12)$$

## THE CONTRAST RATIO

By definition, contrast ratio  $= \frac{B_b}{B_{sb}}$ , which by equations (11) and (12) becomes

$$\frac{B_b}{B_{sb}} = \frac{Af(\theta, \psi) e^{-\rho l} + Z_1 \frac{\alpha}{\rho} (1 - e^{-\rho l})}{Z_1 \frac{\alpha}{\rho} (1 - e^{-\rho l'}) + Z_2 \frac{\gamma}{\sigma} (1 - e^{-\sigma l}) e^{-\sigma \bar{d}} e^{-\rho l'} + Z_1 \frac{\alpha}{\rho} (e^{-\rho l'} - e^{-\rho l}) e^{-\sigma l} + Af(\theta, \psi) e^{-\rho l} e^{-\sigma l}} \quad (13)$$

From the standpoint of forest-fire detection the important quantity is  $l^*$ , the maximum distance that a smoke of defined characteristics can be seen. The distance of the smoke from the observer  $l'$  becomes  $l^*$  when the contrast ratio has the value  $(1 \pm v)$ , in which  $v$  is the threshold of brightness contrast. Making this substitution in (13) and arranging explicitly for  $l^*$  we have

$$l^* = \frac{1}{\rho} \ln \frac{(1 - e^{-\sigma l}) \left( Z_2 \frac{\gamma}{\sigma} e^{-\sigma \bar{d}} - Z_1 \frac{\alpha}{\rho} \right)}{1 \pm v \left[ Af(\theta, \psi) e^{-\rho l} + Z_1 \frac{\alpha}{\rho} (1 - e^{-\rho l}) \right] - Z_1 \frac{\alpha}{\rho} (1 - e^{-\sigma l} e^{-\rho l}) - Af(\theta, \psi) e^{-\rho l} e^{-\sigma l}} \quad (14)$$

Equation (14) symbolizes the maximum distance that a smoke of defined characteristics  $\gamma$ ,  $\Sigma$  and  $t$  can be seen by an eye of normal vision  $v$ , in terms of the haziness of the atmosphere  $\rho$  and  $\alpha$ , the distance  $l$  of the background, the distribution of the incident light and reflection characteristics of the background  $A$ , the inclination  $\psi$  of the background, the direction  $\theta$  of the sun's rays, and two quantities,  $Z_1$  and  $Z_2$ , which are functions of the distribution of the incident and scattered light, respectively for atmospheric haze and the kind of smoke under observation. The cancellation of  $I$  from equation (13) so that it does not appear in (14) indicates that intensity of illumination, within those limits beyond which it may effect  $v$ , has no influence upon the visibility of a smoke.

The preceding discourse has been a purely theoretical development of a complete expression for the visibility of a smoke column in terms of fundamental properties of smoke, haze, and background. If the result (equation 14) were simpler,  $l^*$ , would be immediately calculated by evaluating all quantities on the right-hand side of the equation. This is not feasible in an expression of such complexity, which, for practical use, must first be simplified. In the following simplification certain reasonable approximations are made, indefinite functions are replaced by quantities amenable to experimental determination, and variables of minor importance are omitted entirely from consideration. Further, in order to allow for vagaries of smoke, it is suggested that certain composite functions of variables be evaluated from observations of  $l^*$  made on actual smokes.

#### A SIMPLIFICATION OF EQUATION (14)<sup>7</sup>

The form of equation (14) can be improved by the use of "close-up" brightness ratios as follows:

##### ELIMINATION OF $Z_1$

In figure 3, if the eye be directed towards the horizon sky, the flux to enter the pupil would be expressed by equation (4) with  $l$  set at infinity, namely,  $Z_1 I da \Delta\omega \frac{\alpha}{\rho}$ . This quantity, divided by  $da \Delta\omega$ , is

the horizon brightness,  $B_h$ .

$$B_h = Z_1 I \frac{\alpha}{\rho} \quad (15)$$

##### ELIMINATION OF $Z_2$ , $\sigma$ , $\gamma$ , AND $l$

The light flux from the direction of the smoke reaching an observer standing close in front of the smoke would be  $\frac{d\phi_2 + d\phi_3 + d\phi_5}{e^{-\rho l'}}$ . Hence, from equations (8), (6), and (3),  $B_{sb}$ , the brightness of a smoke viewed at close range against a terrestrial background, would be

$$B'_{sb} = Z_2 I \frac{\gamma}{\sigma} (1 - e^{-\sigma t}) e^{-\sigma d} + Z_1 I \frac{\alpha}{\rho} e^{-\sigma t} \frac{(e^{-\rho l'} - e^{-\rho l})}{e^{-\rho l'}} + I A f(\theta, \psi) \frac{e^{-\rho l}}{e^{-\rho l'}} e^{-\sigma t} \quad (16)$$

<sup>7</sup> The writer is indebted to Deane B. Judd of the Colorimetry Section of the Bureau of Standards, Washington, D. C., for suggesting the use of the conception of apparent luminous reflectance and "close-up" brightness ratios employed in the simplification of equation (14).



ELIMINATION OF  $Af(\theta, \psi)$ 

When the background is viewed at close range,  $B_b$  becomes  $B'_b$  and equation (11), for  $l=0$ , becomes

$$B'_b = IAf(\theta, \psi) \quad (17)$$

Substituting from (15), (16), and (17) in equation (13) the expression for the contrast ratio becomes

$$\frac{B_b}{B_{sb}} = \frac{B'_b e^{-\rho l} + B_h(1 - e^{-\rho l})}{B_h(1 - e^{-\rho l'}) + B'_{sb} e^{-\rho l'}} \quad (18)$$

and equation (14) becomes

$$l^* = \frac{1}{\rho} \ln \frac{1 - \frac{B'_{sb}}{B_h}}{1 - \frac{1}{1 \pm v} \left( \frac{B'_b}{B_h} e^{-\rho l} + 1 - e^{-\rho l} \right)} \quad (19)$$

ELIMINATION OF  $l$ 

For practical purposes it would seem possible to eliminate one more variable by assuming the background to be always a certain small distance beyond the smoke, as, for instance, 0.1 mile for smokes on the near side of a mountain ridge, and 3 miles for smokes on the crest or remote side of a ridge, values best determined by field study of each locality. This having been done, equation (19) could be written in the form:

$$l^* = \frac{1}{\rho} \ln \frac{\left( \frac{B'_b}{B_h} - 1 \right) e^{-\rho(l-l^*)} + \left( 1 - \frac{B'_{sb}}{B_h} \right) (1 \pm v)}{\pm v} \quad (20)$$

in which  $l-l^*$  would be assigned the particular value decided upon.

Equation (20) is a pleasing simplification of equation (14). It implies that when  $v$  and  $l-l^*$  have been established for actual field conditions, the maximum distance that a given smoke could be seen depends upon three quantities  $\rho$ ,  $\frac{B'_b}{B_h}$ , and  $\frac{B'_{sb}}{B_h}$ , functions of the haze, of the background, of the smoke, and of the manner of illumination. It leaves unsolved the difficult problem of evaluating the close-up ratios in terms of the nature of the background, the haze, the smoke, the illumination, and the sun's azimuth.

## VISIBILITY DISTANCE OF SMOKE AGAINST THE HORIZON

In the foregoing, the lookout has been considered to be observing the smoke against a mountainous background. The smoke may of course be viewed against the sky. Any formula derived above can be changed to meet this alteration of conditions by setting  $B'_b = B_h$  and  $l = \infty$ .

$B'_b$  then becomes  $B'_{sh}$ , and  $B_{sb}$  becomes  $B_{sh}$ .

Equation (14) for horizon background becomes

$$l^* = \frac{1}{\rho} \ln \frac{(1 - e^{-\sigma l}) \left( Z_2 \frac{\gamma}{\sigma} e^{-\sigma \bar{a}} - Z_1 \frac{\alpha}{\rho} \right)}{Z_1 \frac{\alpha}{\rho} \frac{\pm v}{1 \pm v}} \quad (21)$$

Similarly, equation (20) takes the form

$$l^* = \frac{1}{\rho} \ln \frac{(1 \pm v) \left(1 - \frac{B'_{sh}}{B_h}\right)}{\pm v} \quad (22)$$

In terms of the fundamental quantities,  $B'_{sh} = \frac{d\phi_2 + d\phi_3}{da \Delta \omega}$  with  $l' = 0$  and  $l = \infty$ , or

$$B'_{sh} = Z_2 I_\sigma^\gamma (1 - e^{-\sigma t}) e^{-\sigma \bar{a}} + Z_1 I_\sigma^\alpha e^{-\sigma t} \quad (23)$$

which shows  $B'_{sh}$  to depend on illumination and on characteristics of the smoke and haze.

#### VISIBILITY AND APPARENT SIZE OF SMOKE COLUMN

No mention has been made of the size of the smoke, although the maximum distance a smoke can be seen depends on its apparent dimensions, particularly the subtended angular width. It would seem possible to allow for size as a formal variable by considering that, whereas  $v$  may be constant for neutral objects of large to moderate apparent size, it is not constant for objects of small subtended angular dimensions. Indeed,  $v$  is appreciably larger above 2 minutes and must be rather great for objects approaching the minimum angle of visual resolution. Moreover, these small angles may be especially important in forest-fire smoke detection.

Under good laboratory conditions,  $v$  is often taken to be 0.02, but actually it is a many-functioned variable dependent upon illumination, glare, adaptation of the eye, sharpness of image, as well as size and shape of object. Consequently, it would seem necessary to determine  $v$  from actual smokes under typical field conditions and thus to relate  $v$  experimentally to apparent size. In this way  $v$  would be expressed as a function of the subtended width,  $t/l^*$ , perhaps as an empirical equation of the form

$$v = p(t/l^*)^{-q} + r \quad (24)^8$$

After evaluation, equation (24) may be utilized to replace  $v$  in (14) and (20) with its equivalent function in terms of  $t$  and  $l^*$ . The resulting equations would involve  $l^*$  as the only unknown and would be solutions of the problem for both size of smoke and brightness of smoke.

A telescope has the effect of increasing apparent dimensions without altering relative brightnesses. It might be argued that binoculars could be used to increase the subtended angular width to  $m \frac{t}{l^*}$  (in which  $m$  is the magnification) and to extend correspondingly the

<sup>8</sup> The constants  $p$ ,  $q$ , and  $r$  in equation (24) may be evaluated approximately to give

$$v = 23 \cdot 10^{-8} (t/l^*)^{-1.72} + 0.03$$

from the data of Helmholtz as presented by Middleton (10, p. 32). This equation holds for Byram's observations ( $t$ ) on smoke,  $v$  being obtained by applying equation (14) to his data and conditions, on the assumption that the diameter of the smoke body at the time of observation varied regularly with distance from 8 to 12 feet. Assuming a constant diameter of 10 feet, Byram's data lead to the relation,

$$v = 0.021 + 1.18 \cdot 10^{-8} (l^*)^2.$$

radius of vision. In practice, however, binoculars are rarely used to detect forest-fire smokes. Rather are they used to identify a smoke as such after it has been discovered. A magnification of  $m$  times is not the equivalent of an  $m$ fold increase in smoke diameter. The former increases visibility through increase in subtended angle, the latter not only through increase in subtended angle but also through increase in brightness because of greater smoke depth along the line of sight.

#### SMOKE VISIBILITY AND WIND

Visibility of the smoke from an incipient forest fire is influenced to an important extent by wind intensity and turbulence, velocity gradient, and wind direction relative to the observer-smoke axis. This influence is quite complex and is exerted through increase in the rate of burning, control of the completeness of combustion and of the amount and quality of suspensoid, dispersion of the suspensoid and lowering of its concentration, change in the size and volume of the smoke, and alteration of the geometrical shape and the depth through which the observer sights. The effect of wind is thus upon size  $t$ , optical concentration  $\sigma$ , and quality  $\frac{\gamma}{\sigma}$ . Since  $l^*$  has been expressed in terms of these quantities, wind as a separate variable has not entered into the theory. Nevertheless, in practical experimentation upon smoke visibility, in view of the impossibility of producing a smoke column always of given diameter, concentration, and quality and of the difficulty of measuring these quantities, it may prove expedient to evolve suspensoid at a constant rate by artificial control and then to ascertain the effect of wind on the visibility of the smoke thus produced. This procedure will be discussed below under proposal III for the estimation of smoke-visibility distance.

#### THE "STANDARD SMALL SMOKE"

To the question, "How far can a smoke be seen?" no answer can be given without first asking, "What kind of a smoke?" Throughout the above discussion we have constantly employed such phrases as "a given smoke" or "a smoke of defined characteristics." By given or defined is meant that the values for  $\gamma$ ,  $\sigma$ , and  $t$  of the smoke are known or that the quality, concentration, and size have been otherwise established.

It has been customary in experimental work on smoke to employ uniformly-formulated smoke compounds in the form of candles or pots to evolve a smoke or fume which is referred to as the "standard small smoke." Although a single arbitrary smoke can fulfill only one set of  $\gamma$ ,  $\sigma$ , and  $t$  values, and the permissibility of taking this "standard smoke" as a unit of measurement is questionable, it is both simple and convenient to express visibility as the distance at which such a standard small smoke can be seen.

Although the standard small smoke must be chosen arbitrarily, to be serviceable it should be made to duplicate in appearance typical smokes as they are first detected in fire-protection practice. This duplication must be in respect to all the properties of smoke that enter into equation (14), namely size,  $t$ , optical concentration,  $\sigma$ ,

quality (largely sootiness),  $\frac{\gamma}{\sigma}$ , and angular scattering function,  $Z_2$ .

If the standard smoke chosen be too small, its visibility may be determined almost wholly by subtended angular width; such factors as position of the sun and optical density of the haze, which ordinarily are very important, may be insignificant. In other words, the chosen smoke must be typical in size and concentration; it must not be whiter or darker, heavier or lighter, than the just-detected smokes from the forest fuel of chief interest; and it must look like the latter from all directions. If these conditions are met the calculated visual distances will be of the same order of magnitude as distances encountered in practice.

#### ESTIMATION OF SMOKE VISIBILITY DISTANCE

For the practical utilization of this theory of smoke visibility, several procedures suggest themselves. In equation (14), 12 independent variables compose the maximum visibility distance,  $l^*$ . Limiting consideration to a standardized smoke reduces these 12 to 9. Not all 9 are of equal importance and some may be neglected, taken as constant, or expressed only qualitatively without greatly increasing the standard error in the estimation of  $l^*$ . It would seem possible to avoid the complication of 2 of these 9 ( $l$  and  $v$ ) by the following expedients.

Equation (14) may be thrown into the following form:

$$l^* = \frac{1}{\rho} \ln \left[ e^{-\rho(l-l^*)} \left( \frac{Af(\theta, \psi) - I}{Z_1 \alpha / \rho} \right) \left( \frac{1 - e^{-\sigma t(1 \pm v)}}{\pm v} \right) + \left( 1 - \frac{Z_2}{Z_1} \frac{\gamma}{\sigma} \frac{\rho}{\alpha} (1 - e^{-\sigma t}) e^{-\sigma d} \dots e^{-\sigma t} \right) \frac{1 \pm v}{\pm v} \right] \quad (25)$$

in which  $l$  is introduced as the difference ( $l-l^*$ ), which better elucidates its physical role. Between the smoke column and the background there exists illuminated haze scattering light to the eye of the observer, imposing added brightness upon the background, and diminishing the contrast ratio. In forest-fire-detection systems, lookouts are so stationed that the major portion of the forest area is in direct view. Accordingly, smokes are usually seen with rising ground or ridges as backgrounds. Under these circumstances ( $l-l^*$ ) would be small, possibly negligible, and could often be taken as constant without much error.

Instead of measuring every independent factor and then calculating the dependent variable ( $l^*$ ), it is desirable to observe the latter directly, in order empirically to evaluate functions and quantities that do not easily lend themselves to direct measurement. To observe  $l^*$  in this way has the advantage of automatically including the function of the vision  $v$  and introducing its effect into all empirical relationships so determined. This is the method advocated in all but the final proposal listed below.

In these proposals are successively included the more important variables, leading progressively to increasing complexity but to diminishing standard error in the estimation of  $l^*$ . That proposal should be chosen which fulfills the required accuracy without too great complication.

I. Equation (14) might be reduced in form to  $l^* = \frac{P}{\rho}$  in which  $P$  is the complex logarithm which, for the purpose of extreme simplification, would be taken as a constant and evaluated by observing the distance ( $l^*$ ) that the standard small smoke could be seen through a hazy atmosphere of attenuation coefficient  $\rho$ . In view of the dependence of the more distant observations upon  $v$ , as explained above, it would be more rational to plot the data in the form  $l^*$  vs.  $\frac{1}{\rho}$  and thus to allow for the possibility of finding  $\rho l^*$  somewhat less at high values of  $l^*$  than at low values.

This plot might be made for two conditions, smoke in sunlight and smoke in shade.

The coefficient,  $\rho$ , can be evaluated by a hazemeter (3) which on the basis of the Koschmieder (6) (7) equation,

$$\frac{B_b}{B_h} = 1 - e^{-\rho l},$$

can be used to measure the ratio of the horizon brightness to the brightness of a black target,  $l$  miles away; or, following the suggestion and equation of Löhle (8),

$$\frac{B_1}{B_2} = \frac{1 - e^{-\rho l_1}}{1 - e^{-\rho l_2}}$$

the brightness ratio of two black targets at different distances,  $l_1$  and  $l_2$  from the observer. Both equations assume uniform haze and uniform illumination over the line of sight.

II. Equation (14) might be rewritten as  $l^* = \frac{Q}{\rho}$  in which  $Q$  is assumed

to be a function of  $\theta$  only.  $\theta$  is the supplement of the angle between observer, smoke, and sun, the smoke being at the vertex. Since the sun's rays are parallel,  $\theta$  is also the angle between sun, observer, and smoke, with the observer at the vertex. Of the several variables on which  $Q$  actually depends,  $\theta$  is chosen as one of primary importance. From this suggestion,  $l^*$ ,  $\rho$ , and  $\theta$  would be determined for the standard small smoke. From these data  $Q (= \rho l^*)$  would be plotted in graphic relation to  $\theta$ , or preferably, because of the possible effect of  $v$ ,  $l^*$  against  $\theta$  with  $\rho$  as a third variable. Thereafter, with  $\theta$  and  $\rho$  known,  $l^*$  for the standard small smoke could be estimated from this plot to the degree of accuracy allowed by the above assumption. This plot might be made for smoke in shade as well as for smoke in sunlight.

III. It is probable that the standard error of proposal II would still be too high for reasonable satisfaction and that the independent variables of next greatest importance should be included. These may

be  $t$ ,  $\sigma$ , and  $\frac{\gamma}{\sigma}$  which, despite the fact that a standard smoke is being

considered, are not constant but are influenced by wind which acts directly on size, shape, and density of the smoke column.<sup>9</sup> In view

<sup>9</sup> Air temperature and relative humidity have effects on smoke, but for atmospheres of the forest-fire season well above the dew point, they may not be of major importance.

of the influence of the wind, equation (14) could take the form  $l^* = \frac{R}{\rho}$ ,

in which  $R$  would be considered a function not only of  $\theta$  but also of  $w$ , the wind intensity. In this case, experimental data on the standard small smoke would be gathered for  $l^*$ ,  $\rho$ ,  $\theta$ , and  $w$  and a plot made of  $R$  ( $=\rho l^*$ ) against  $\theta$  for various wind intensities, as illustrated in figure 5 (in which the curves are drawn "wavy" to indicate that their true forms are as yet unknown).

As mentioned in the discussion of smoke visibility and wind, the direction of the wind relative to the observer's line of sight is important. A smoke being blown along this axis would undoubtedly be more clearly visible than the same smoke moving across the axis. Limitation of experimental observations to smoke in the cross wind, as far as practicable, would make the estimated value of  $l^*$  tend toward a minimum. The alternative would be to determine the data of figure 5 for both cross and longitudinal winds and possibly for intermediate directions.

Furthermore, since the vertical distribution of wind is different for different forest types (open grassland, brush, and woodlands) (5) and since the diffusion of smoke as it rises through the forest canopy is influenced by this vertical distribution, the cover type in which visibility observations are made should be specified.

Since  $\rho l^*$  may vary with  $r$  when all other factors are held constant, preferable to figure 5 would be a three-dimensional figure with  $l^*$  plotted on one axis.

IV. Of the variables affecting visibility distance, procedure III includes only three, and leaves it understood that all variations in background, illumination of background, illumination of smoke and haze, and quality of haze, are relatively immaterial. Of these, the one that should next be considered is probably background, symbolized by  $A$  in equation (14). For practical purposes descriptive terms might be employed such as ponderosa pine woods, yellow grass, gray snag field, brown talus rock, etc. Accordingly, to allow for background differences, the graph shown in III would be considered as limited to one particular type of background.

V. The angle of inclination  $\psi$ , occurring as one of the factors in equation (14), might be expressed in terms of degrees or percent slope. Practically, however,  $\psi$  is of minor importance as long as the earth element is not inclined so far as to approach grazing incidence or to be in its own shadow. A perfectly diffusing surface appears of the same brightness at all inclinations, and to the extent that pine woods, for instance, approach perfect diffusion the less influence has  $\psi$ . Reflection from a pine-covered mountain comes much nearer to being completely diffuse than unidirectional, and it is probable that ordi-

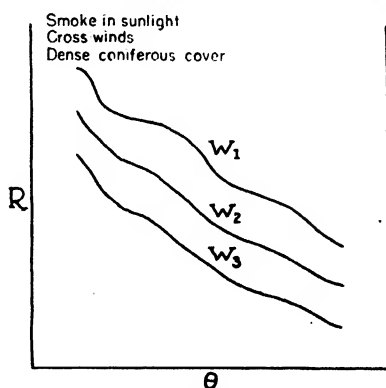


FIGURE 5.—Illustration of the way the relation between  $R$ ,  $\theta$ , and  $w$  may be shown graphically for particular types of illumination, wind direction, and background.

narily the only consideration that need be given to  $\psi$  is to state "Background in sunlight" or "Background in shadow." It is suggested in this procedure that the data for figure 5 be determined for each of these two conditions.

VI. Illumination affects smoke visibility through the quantities  $A$ ,  $Z_1$ , and  $Z_2$ . By "illumination" is meant such natural conditions as (1) bright sunlight in a clear sky, (2) a sky veiled with clouds or mist (light, medium, heavy) through which the direct rays of the sun penetrate only in part, (3) a thickly overcast sky which conceals utterly the position of the sun. In practice it would probably suffice to express illumination in qualitative terms such as the above. To what extent variations in illumination play a part in visibility is uncertain, but if extreme variations are encountered illumination must be considered and figure 5 redetermined for an adequate number of gradations.

VII. To the extent that  $(l-l^*)$  is negligible, the  $\rho$  in the quantity  $e^{-\rho(l-l^*)}$  is also negligible, and haze enters into the logarithm of equation (25) only in the form  $\frac{\alpha}{\rho}$ . In haze of a given quality, the ratio of light absorbed to light scattered  $(\beta/\alpha)$  is a constant, and since  $\rho = \alpha + \beta$ ,  $\frac{\alpha}{\rho}$  also is constant.  $\frac{\alpha}{\rho}$  might be measured and expressed numerically but it would be easier and probably sufficient simply to describe the quality of the haze as "vaporous," "smoky," "dusty," "industrial," or "misty." If the effects of variation in  $\frac{\alpha}{\rho}$  are found to be large, figure 5 should be drawn for each important class of haze.

VIII. Proposal III, with the additions suggested in IV, V, VI, and VII, takes into account all independent variables of equation (14) to provide a complete solution. Equation (20) offers another way to include all variables in estimating visibility distance.

The two close-up brightness ratios in equation (20) are quantities which could be measured experimentally against a nearby black background. According to equations (15), (16), and (17), they are in fundamental terms as follows:

$$\frac{B'_b}{B_h} = \frac{Af(\theta, \psi)}{Z_1 \alpha / \rho} \quad (26)$$

$$\frac{B'_{sb}}{B_h} = \frac{Z_2}{Z_1} \frac{\gamma}{\sigma} \frac{\rho}{\alpha} (1 - e^{-\sigma t}) e^{-\sigma \bar{a}} \quad (27)$$

From these relations, it is evident that for a specified type of illumination ( $A$ ,  $Z_1$ ,  $Z_2$ ) for a given background ( $A$ ,  $\psi$ ) and for a smoke of defined characteristics ( $\gamma$ ,  $\sigma$ ,  $t$ ), the ratios will vary with the sun's

azimuth ( $\theta$ ) and the quality of the haze ( $\frac{\alpha}{\rho}$ ). For a standard small smoke,  $\gamma$ ,  $\sigma$ , and  $t$  will be constant except as they are affected by winds. On this basis it would be possible to prepare graphs of the kind illustrated in figure 6. By measuring  $w$  and  $\theta$  and converting them into their functions by use of the plots of figure 6, by determining  $\rho$ , by

knowing the value of  $v$ , and by assuming a reasonable value for  $(l-l^*)$ , the observer can determine every quantity in equation (20) except  $l^*$ , which is immediately calculable.

This procedure has the advantage of providing a direct test of the validity of the theory by comparison of observed and calculated values of  $l^*$ . Its disadvantages lie in the necessity of establishing the relation between  $v$  and  $t/l^*$  and of predetermining the close-up brightness ratios on that part of the smoke column which is most important in rendering the smoke initially visible.

### DISCOVERY TIME

In an actual forest fire the perimeter is progressively increasing. As a consequence,  $t$  (and possibly  $\sigma$ ) varies with time as well as with all the factors which control the rate of spread of the fire. If  $t$  in equation (14) were replaced with its equivalent function in terms of

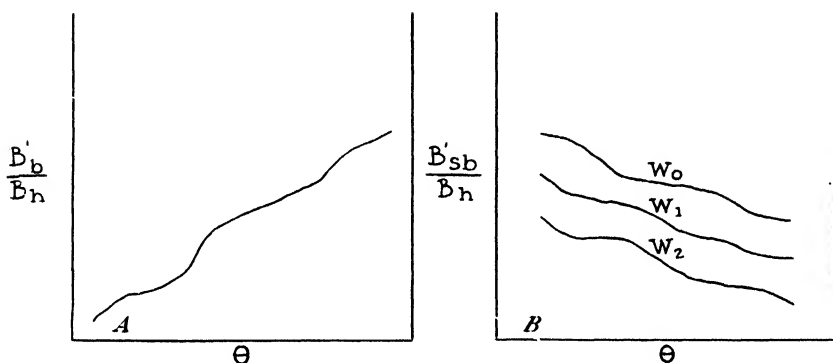


FIGURE 6.—Illustration of the way in which the relation between the close-up brightness ratios and  $\theta$  may be shown graphically for particular conditions of background, illumination, haze, wind, and smoke: *A*, Clear sky, ponderosa pine forest, background in sunlight, smoky haze; *B*, clear sky, cross wind, ponderosa pine forest, standard smoke candle, smoky haze.

time and rate of spread, the result would be an expression connecting distance between lookout and smoke with the minimum time of discovery. Discovery time is thus indicated to be dependent upon  $l^*$ ,  $\rho$ ,  $\theta$ ,  $v$ ,  $l$ , the background brightness, the illumination of the background, the illumination of the smoke, the quality of the haze, the wind direction, the cover type, and the rate of spread of the fire. The time required for a lookout to discover a smoke of rapidly increasing proportions is probably of more practical utility than the radius of vision for a smoke of constant size and density.

### SUMMARY

A theoretical analysis has been given of the visibility of a smoke column from a forest lookout for the purpose of clarifying ideas on the physical basis of smoke detection. The visibility of the smoke is considered to be its contrast in brightness with the background. The contrast ratio is expressed in terms of the light flux entering the observer's eye from the smoke, the haze, and the background and the



flux in terms of the scattering and reflecting properties of the various elements. From this expression an equation is derived for the maximum distance a given smoke could be seen under specified conditions. This equation is simplified by substituting "close-up" brightness ratios for elementary scattering functions and by drawing approximations through the introduction of simple assumptions. The equations appertain to various types of illumination and to smokes seen against both sky and terrestrial background.

The theory indicates that the maximum distance at which a smoke is visible is inversely proportional to the amount of haze in the atmosphere, the proportionality factor being the logarithm of a complex quantity involving the sun's azimuth along with certain characteristics of background, the smoke, the illumination, and the vision.

For the practical utilization of the derived equations there are suggested several procedures of increasing complexity leading to diminishing standard error in the estimation of maximum visibility distances.

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# COMPARISON OF RATES OF APPARENT PHOTOSYNTHESIS AND RESPIRATION OF DISEASED AND HEALTHY BEAN LEAFLETS <sup>1</sup>

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## INTRODUCTION

When a leaf is attacked by a pathogen, it is reasonable to assume that some, if not all, of its physiological processes are affected. Increased respiration and transpiration, delayed translocation, increased or decreased photosynthesis, and an interference with the movement of water and solutes in the vascular system are some of the physiological changes recorded for diseased leaves.

The data presented here consist of comparative measurements of apparent photosynthesis and of respiration on healthy and diseased bean leaflets, respectively. Wherever possible, correlations are indicated between outward symptoms of disease and abnormal physiology.

## REVIEW OF LITERATURE

Reed and Crabill (8)<sup>3</sup> showed that the presence of aecia of *Gymnosporangium juniperi-virginianae* Schw. reduced the carbon dioxide assimilation of apple leaves 56 to 65 percent. Diseased leaves showed more rapid and also greater total respiration than healthy leaves.

Long (7) demonstrated that members of the genera *Puccinia* and *Uromyces* reduce the assimilation rate of oat and wheat leaves by approximately 30 percent. Bailey and Gurjar (1) found that wheat plants infected with *P. graminis tritici* Erikss. and Henn. exhibit a reduced rate of respiration.

Yarwood (13) reported increased respiration of excised clover leaflets infected by *Erysiphe graminis* DC. or by *Uromyces fallens* Kern.

Kuprewicz (6) lists as changes which occur within plants attacked by microorganisms: Decrease in the chlorophyll content and carbon assimilation, increased or decreased respiration, retarded translocation, and increased transpiration.

Smith (9) reviews the literature on the metabolism of virus-diseased plants. Mosaic diseases are accompanied by an increase in the total nitrogen and a decrease in the total carbohydrates; yellows diseases produce the opposite effect. Diseased plants often show a delayed translocation. Respiration rates of leaves are higher than normal with young leaves, and lower than normal with old leaves.

<sup>1</sup> Received for publication March 5, 1940. This paper is an abridgment of a thesis presented to the Graduate School of Cornell University in partial fulfillment of the requirements for the degree of doctor of philosophy.

<sup>2</sup> The writer is indebted to Dr. L. M. Massey, under whose guidance the work was undertaken, for his continued interest and help throughout the investigation. The many timely suggestions of Dr. A. J. Heinicke are gratefully acknowledged.

<sup>3</sup> Italic numbers in parentheses refer to Literature Cited, p. 191.

## MATERIAL AND METHODS

The writer sought a plant, susceptible to plant pathogens and adaptable to greenhouse cultivation, which produces its leaves in opposite pairs on a common stem. The bean plant (*Phaseolus vulgaris* L.) fulfills these requirements, and a pure line of the variety Red Kidney was selected for study.

The first leaves of the bean plant are simple; the second and succeeding leaves are compound and consist of three leaflets, one terminal and a pair of opposite leaflets. In area, opposite leaflets are very similar, and since they possess a common petiole, their nutrition should also be very similar. It was therefore decided to study pairs of opposite leaflets, comparing the physiology of one member of a pair with that of the other. The following comparisons are presented here: (1) Between the physiology of the two members of a pair of healthy opposite leaflets; (2) between the physiology of a pair of opposite leaflets, one member diseased, the other healthy; and (3), between the physiology of the two members of a pair of diseased opposite leaflets.

Apparent photosynthesis was measured as the amount of carbon dioxide utilized by the bean leaflets, and respiration as the amount of carbon dioxide given off by the leaflets. Air of known carbon dioxide content was passed over the leaflets enclosed singly in envelopes made from heavyweight moisture-proof cellophane, with the edges sealed with Du Pont moisture-proof adhesive No. 395. The air was then bubbled through absorption towers filled with potassium hydroxide. The apparatus used was similar to that developed by Heinicke and Hoffman (4) in their studies on the rate of photosynthesis of apple leaves under natural conditions. The difference between the carbon dioxide content of the stream of air passed over the plant material and a comparable stream of normal air determined the photosynthesis of respiration.

Special leaf holders were devised which combined the features of support and air intake. They consisted of two pieces of glass tubing, bent and pushed through a rubber stopper, and held in position by a burette clamp and ring stand. A pair of leaf holders and a pair of opposite bean leaflets enclosed in cellophane envelopes are shown in figure 1.

Bean plants were grown in the greenhouse until the first compound leaf was approximately half its mature size. The plants were then transferred to a darkened room with illumination provided by a pair of 1,000-watt bulbs complete with reflectors, supplying 800 foot-candles at leaf level, 20 inches distant from the source of light. The length of illumination was arbitrarily fixed at 13 hours, from 4:00 a. m. to 5:00 p. m. Plants were kept in this room for 48 hours before physiological measurements were started, and continuously thereafter until studies were terminated.

While the room temperature was fairly constant (72° to 75° F.), that of the air at leaflet level varied from 83° to 88° F. during hours of illumination, occasionally reaching 90°. It is of interest to note that Yoshii (14) records greatest assimilation by *Phaseolus* spp. in strong light at 96.8°; decreasing the light intensity reduced the optimum temperature to 80.6°. No measurements were taken of air temperatures within cellophane containers in the present investigation.

Air was supplied to the leaflets at the rate of 2 to 2.5 liters per square centimeter of leaflet surface per hour, as recommended by Heinicke and Hoffman (4). The volume of air passed over the plant material was calculated from three readings taken with an air-flowmeter during each 4-hour determination. The carbon dioxide content of the air fluctuated from day to day and, occasionally, during a single day, varying from 0.72 to 0.85 mg. per liter during these studies. The plants were grown in greenhouse soil and watered daily with distilled water.

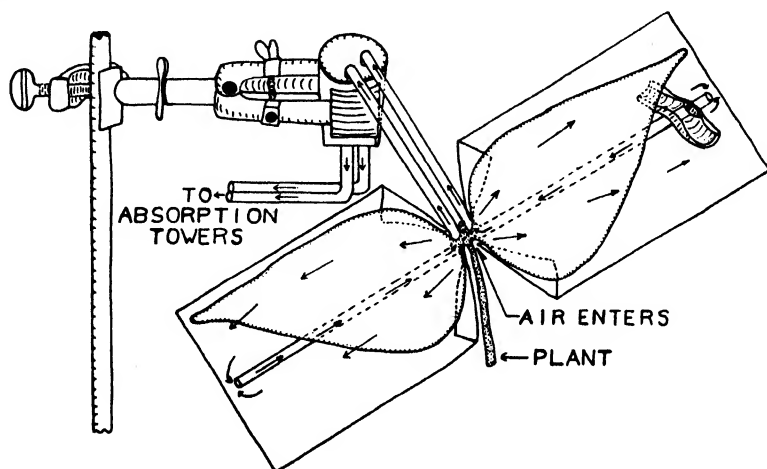


FIGURE 1.— Pair of leaf holders in position, with a pair of opposite bean leaflets enclosed in cellophane envelopes. The path of the air stream is indicated. The third leaflet of the compound leaf of the bean plant is not shown.

A number of investigators have reported the effect of maturity on the physiology of plant tissue. Spoehr (11) cites the investigations of Willstätter and Stoll, who showed that photosynthesis was quite high in leaves that were just unfolding, the rate becoming constant after about 9 days of growth. With further aging the rate decreased. Kidd et al. (5) showed that respiration of *Helianthus annuus* decreased with maturity. Héc (3) found a decrease in respiration with increase in age. Stiles (12), discussing the effect of aging on the activity of a leaf, says that "as a leaf gets older, the chlorophyll content increases and along with this the rate of photosynthesis, but not at the same rate. Hence with increase in chlorophyll content the assimilation number falls." To reduce the variable of leaflet age, the first physiological measurements were begun 13 to 15 days after the seed was planted, and data were taken during successive 5-day periods. Data taken on the first pair of leaflets on a plant during the first 5 days were designated "period A" data, data during the following 5 days were designated "period B" data, data taken during the next 5 days were designated "period C" data, and so on. Data on other leaflets on the same plant were similarly arranged; for example, period A for the second pair of leaflets was period B for the first pair, and period A for the third pair was period C for the first pair. By the use of this scheme, it was possible to group together data obtained from leaflets of comparable physiological maturity.

One or two determinations of 4 hours each on apparent photosynthesis were made daily, between the hours of 8:00 a. m. and 12:30 p. m., and between the hours of 12:30 p. m. and 5:00 p. m. One 4-hour measurement of respiration was made daily between 7:00 p. m. and midnight with the plants in the dark. Measurements on apparent photosynthesis taken between 8:00 a. m. and 12:30 p. m. are designated "a. m." data in all tables; those obtained between 12:30 p. m. and 5:00 p. m. are designated "p. m." data.

Data were calculated as milligrams of carbon dioxide assimilated or respired per 100 cm.<sup>2</sup> of leaflet surface per 4-hour determination. Leaflet areas were obtained from planimeter measurements of contact photographic prints, taken of each leaflet at 3-day intervals. The method of analysis of variance (10) was used in the biometrical analysis of all data.

## EXPERIMENTAL RESULTS

### PHYSIOLOGY OF PAIRS OF HEALTHY LEAFLETS

#### APPARENT PHOTOSYNTHESIS

Seventeen pairs of leaflets, on 10 different bean plants, were studied: 3 pairs on each of 3 plants, 2 on 1, and 1 pair on each of 6 others.

TABLE 1.—Mean assimilation rates of pairs of healthy, opposite bean leaflets at three periods of maturity, expressed as milligrams of carbon dioxide per 100 cm.<sup>2</sup> of leaflet surface per 4-hour determination

[Each mean is the average of 8 to 10 individual observations]

Pair of leaflets No.	Leaflet No.	Mean $\pm$ S. E. assimilation rates of leaflets at indicated period of maturity <sup>1</sup>		
		Period A	Period B	Period C
		Milligrams	Milligrams	Milligrams
1	7		48.3 $\pm$ 1.9	
	17		45.7 $\pm$ 1.9	
2	11		27.0 $\pm$ 2.3	
	21		29.1 $\pm$ 2.3	
3	32		39.7 $\pm$ 3.3	
	42		37.7 $\pm$ 3.3	
4	37		24.7 $\pm$ 3.1	
	47		26.9 $\pm$ 3.1	
5	77	46.7 $\pm$ 3.3		50.0 $\pm$ 3.0
	87	39.0 $\pm$ 3.3		46.5 $\pm$ 3.0
6	110	22.6 $\pm$ 4.6		
	210	26.2 $\pm$ 4.6		
7	111	35.7 $\pm$ 4.4		
	211	36.3 $\pm$ 4.4		
8	312	35.8 $\pm$ 4.9		
	412	30.1 $\pm$ 4.9		
9	313	24.7 $\pm$ 4.9		
	413	25.6 $\pm$ 4.9		
10	314	32.8 $\pm$ 4.4		52.3 $\pm$ 5.6
	414	37.7 $\pm$ 4.4		51.6 $\pm$ 5.6
11	713		44.8 $\pm$ 3.1	
	813		43.5 $\pm$ 3.1	
12	714	23.6 $\pm$ 4.6		
	814	27.3 $\pm$ 4.6		
13	715	34.1 $\pm$ 4.4		
	815	34.6 $\pm$ 4.4		37.6 $\pm$ 4.4
14	716	39.5 $\pm$ 5.6		33.6 $\pm$ 4.4
	816	42.5 $\pm$ 5.6		44.7 $\pm$ 0.7
15	913	29.6 $\pm$ 4.1		46.0 $\pm$ 9.7
	1013	28.3 $\pm$ 4.1		
16	914	36.1 $\pm$ 4.4		
	1014	28.6 $\pm$ 4.4		46.3 $\pm$ 4.4
17	915	48.5 $\pm$ 4.6		38.1 $\pm$ 4.4
	1015	56.7 $\pm$ 4.6		52.7 $\pm$ 4.4
Average		34.0 $\pm$ 1.7	37.1 $\pm$ 2.9	45.9 $\pm$ 2.1

<sup>1</sup> Description in text, p. 181.

A few pairs were members of the second, third, and occasionally the fourth compound leaf, but the majority of the pairs studied were those of the first compound leaf. Data on apparent photosynthesis are presented in table 1, with each figure the mean of 8 to 10 individual 4-hour observations.

The results given in table 1 show differences as high as, or higher than, 15 percent between the assimilation rates of the members of a pair of healthy, opposite leaflets. These differences are not statistically significant. Some of the differences between assimilation rates of leaves of comparable physiological maturity (periods A, B, or C) are significant.

In the present studies, the effect of maturity of a bean leaflet was marked. The leaflets were studied for approximately 15 days, and the assimilation rate was highest during the last 5-day interval (period C), even though the data were calculated on a unit-area basis. It is not known whether the maximum rate of assimilation was reached; with further increase in age, the rate should decrease steadily until abscission occurs.

No data were obtained on the respiration rates of pairs of opposite healthy leaflets.

#### PHYSIOLOGY OF PAIRS OF LEAFLETS

##### ONE LEAFLET HEALTHY, THE OTHER DISEASED

Thirteen pairs of leaflets, one member healthy and the other diseased, were studied. Anthracnose, a disease easily reproduced under controlled conditions, caused by *Colletotrichum lindemuthianum* (Sacc. and Magn.) Bri. and Cav., was selected, and the gamma strain (2) used throughout the investigations. The fungus produces necrotic spots on both surfaces of the bean leaflet, but more predominantly on the lower surface. The veinal system is invariably attacked, though interveinal lesions are not uncommon. Secondary symptoms include loss of chlorophyll, desiccation of a part or all of a leaf, and sometimes death, especially when the leaflet is infected in a juvenile stage.

Preliminary studies failed to show a correlation between the amount of inoculum and the severity of subsequently developed symptoms. A spore suspension, which appeared slightly cloudy when held to the light, was applied to both surfaces of the leaflets with a camel's-hair brush. One member of each pair of leaflets was inoculated, the other member similarly treated except that only sterilized water was used. Inoculated plants were well watered, placed for 36 to 48 hours in a moist chamber at approximately 75° F., and then transferred to the illumination chamber. Symptoms usually appeared within 24 to 72 hours after inoculation and measurements of apparent photosynthesis or respiration or both were started immediately following transfer to the chamber, or as soon thereafter as convenient.

#### GROWTH RATES

Bean leaflets affected with anthracnose were occasionally visibly smaller than healthy companion leaflets. Measurements on area were taken at 3-day intervals on 41 pairs of healthy opposite leaflets, and on 21 pairs of opposite leaflets with one member healthy and the other diseased, to determine whether retardation in growth was a

constant symptom following infection with *Colletotrichum lindemuthianum*. Inoculation with the fungus was performed immediately following the initial measurements. Average findings are presented in table 2.

TABLE 2.—Mean percentage increase in the area of members of pairs of healthy opposite leaflets and of members of pairs of opposite leaflets with one member healthy and the other infected with *Colletotrichum lindemuthianum*

[Results from 41 pairs of healthy opposite leaflets and 21 pairs of opposite leaflets with 1 member healthy and 1 diseased]

Condition of members of pairs of opposite leaflets	Approximate time between initial and final measurements	Mean increase in area $\pm$ S. E.	Difference $\pm$ S. E.
	Days	Percent	Percent
Healthy.....	25	81.84 $\pm$ 1.82	
Do.....	25	77.85 $\pm$ 1.82	3.99 $\pm$ 2.57
Do.....	12	18.96 $\pm$ .97	
Diseased.....	12	13.40 $\pm$ .97	5.56 $\pm$ 1.37

<sup>1</sup> Leaflets inoculated when past juvenile stage, hence the percentage increases in area are lower than those given for pairs of healthy leaflets.

The results showed that between the members of pairs of healthy opposite leaflets there was no significant difference in percentage increase in area ( $3.99 \pm 2.57$ ), but between the members of pairs of opposite leaflets with one member healthy and one diseased there was a significant difference in percentage increase in area ( $5.56 \pm 1.37$ ). The difference between these two figures, approximately 1.6 percent, represents the growth restriction due to infection by *Colletotrichum lindemuthianum*.

#### APPARENT PHOTOSYNTHESIS

Data obtained on apparent photosynthesis of the pairs of opposite leaflets studied, the approximate time elapsing between planting of seed and inoculation of leaflet and between inoculation and first measurement of apparent photosynthesis, are given in table 3. The leaflets were under observation for varying lengths of time depending on symptom development and intensity, and on convenience of observation. A single 4-hour reading per day, between the approximate hours of 8:00 a. m. and 12:30 p. m., was taken on the majority of the pairs of leaflets. Data are given as mean assimilation rates, each mean representing the average of three to five individual 4-hour readings. The relation of age to the assimilation rate is indicated by separation of data into periods as was done in table 1.

The figures for mean assimilation when all data are combined are as follows: For healthy leaflets,  $26.29 \pm 1.04$ ; for diseased leaflets,  $19.99 \pm 1.04$ . The difference is  $6.30 \pm 1.48$ .

The majority of the inoculated leaflets showed a reduced rate of assimilation as compared with healthy companion leaflets, but only two leaflets, 36 and 636, showed individual reductions large enough to be within the range of significance. However, when the mean assimilation rate of diseased leaflets is compared with the mean assimilation rate of healthy leaflets for all of the pairs, a 24-percent reduc-

TABLE 3.—Mean assimilation rates of pairs of opposite bean leaflets at five periods of maturity, when one member was healthy and the other was infected with *Colletotrichum lindemuthianum*, expressed as milligrams of carbon dioxide per 100 cm.<sup>2</sup> of leaflet surface per 4-hour determination

[Each mean for the different periods of maturity is the average of three to five observations]

Pair of leaflets No.	Leaflet No. and condition	Time between sowing of seed and inoculation	Time between inoculation and first measurement	Mean assimilation rates in morning and afternoon of leaflets at indicated period of maturity <sup>1</sup>								Mean of all observations $\pm$ S. E.	Difference $\pm$ S. E.
				Period A		Period B		Period C	Period D	Period E			
				P. m. <sup>3</sup>		A. m. <sup>2</sup>							
				Milli-grams	Milli-grams	Milli-grams	Milli-grams						
1.	{ 114, diseased 124, healthy	14	3	{ 20.1 (1) 25.3 (1)	{ 20.1 (1) 22.3 (1)	{ 1.8 (1) 20.1 (1)	{ 29.9 (3) 42.4 (2)	{ 35.6 (3) 55.0 (3)	{ 36.3 (3) 37.7 (3)	{ 19.2 $\pm$ 3.4 24.4 $\pm$ 3.4	{ 5.2 $\pm$ 4.8 10.2 $\pm$ 4.2		
2.	{ 36, diseased 46, healthy	14	3	{ 36.7 (1) 42.8 (1)	{ 37.8 (1) 49.0 (1)	{ 8.7 (2) 12.0 (2)	{ 20.1 (1) 25.3 (1)	{ 29.9 (3) 42.4 (2)	{ 35.6 (3) 55.0 (3)	{ 36.3 (3) 37.7 (3)	{ 19.2 $\pm$ 3.4 24.4 $\pm$ 3.4		
3.	{ 78, diseased 88, healthy	14	3	{ 25.3 (0) 30.7 (0)	{ 29.0 (0) 28.8 (0)	{ 25.3 (1) 28.3 (1)	{ 29.9 (3) 42.4 (2)	{ 35.6 (3) 55.0 (3)	{ 36.3 (3) 37.7 (3)	{ 19.2 $\pm$ 3.4 24.4 $\pm$ 3.4	{ 5.2 $\pm$ 4.8 10.2 $\pm$ 4.2		
4.	{ 108, diseased 108, healthy	14	3	{ 25.8 (1) 34.7 (1)	{ 23.1 (1) 34.7 (1)	{ 10.7 (1) 14.3 (1)	{ 29.9 (3) 42.4 (2)	{ 35.6 (3) 55.0 (3)	{ 36.3 (3) 37.7 (3)	{ 19.2 $\pm$ 3.4 24.4 $\pm$ 3.4	{ 5.2 $\pm$ 4.8 10.2 $\pm$ 4.2		
5.	{ 600, diseased 600, healthy	13	4	{ 25.4 (1) 19.1 (1)	{ 27.8 (1) 31.0 (1)	{ 13.2 (3) 17.8 (3)	{ 29.9 (3) 42.4 (2)	{ 35.6 (3) 55.0 (3)	{ 36.3 (3) 37.7 (3)	{ 19.2 $\pm$ 3.4 24.4 $\pm$ 3.4	{ 5.2 $\pm$ 4.8 10.2 $\pm$ 4.2		
6.	{ 612, diseased 612, healthy	15	4	{ 28.8 (1) 37.6 (1)	{ 29.2 (1) 31.0 (1)	{ 15.3 (3) 17.8 (3)	{ 29.9 (3) 42.4 (2)	{ 35.6 (3) 55.0 (3)	{ 36.3 (3) 37.7 (3)	{ 19.2 $\pm$ 3.4 24.4 $\pm$ 3.4	{ 5.2 $\pm$ 4.8 10.2 $\pm$ 4.2		
7.	{ 614, diseased 615, healthy	14	4	{ 9.4 (1) 21.7 (1)	{ 15.3 (2) 23.9 (2)	{ 23.5 (3) 27.7 (3)	{ 29.9 (3) 42.4 (2)	{ 35.6 (3) 55.0 (3)	{ 36.3 (3) 37.7 (3)	{ 19.2 $\pm$ 3.4 24.4 $\pm$ 3.4	{ 5.2 $\pm$ 4.8 10.2 $\pm$ 4.2		
8.	{ 620, diseased 621, healthy	15	4	{ 33.7 (1) 41.0 (1)	{ 35.1 (2) 37.7 (2)	{ 28.0 (2) 34.1 (2)	{ 29.9 (3) 42.4 (2)	{ 35.6 (3) 55.0 (3)	{ 36.3 (3) 37.7 (3)	{ 19.2 $\pm$ 3.4 24.4 $\pm$ 3.4	{ 5.2 $\pm$ 4.8 10.2 $\pm$ 4.2		
9.	{ 622, diseased 623, healthy	13	4	{ 0.3 (0) 21.7 (1)	{ 27.0 (1) 30.7 (1)	{ 21.5 (1) 25.1 (1)	{ 29.9 (3) 42.4 (2)	{ 35.6 (3) 55.0 (3)	{ 36.3 (3) 37.7 (3)	{ 19.2 $\pm$ 3.4 24.4 $\pm$ 3.4	{ 5.2 $\pm$ 4.8 10.2 $\pm$ 4.2		
10.	{ 634, diseased 635, healthy	15	8	{ 5.3 (1) 18.8 (1)	{ 13.6 (2) 18.4 (2)	{ 16.3 (3) 20.4 (3)	{ 29.9 (3) 42.4 (2)	{ 35.6 (3) 55.0 (3)	{ 36.3 (3) 37.7 (3)	{ 19.2 $\pm$ 3.4 24.4 $\pm$ 3.4	{ 5.2 $\pm$ 4.8 10.2 $\pm$ 4.2		
11.	{ 636, diseased 637, healthy	15	5	{ 5.3 (1) 18.8 (1)	{ 13.6 (2) 18.4 (2)	{ 16.3 (3) 20.4 (3)	{ 29.9 (3) 42.4 (2)	{ 35.6 (3) 55.0 (3)	{ 36.3 (3) 37.7 (3)	{ 19.2 $\pm$ 3.4 24.4 $\pm$ 3.4	{ 5.2 $\pm$ 4.8 10.2 $\pm$ 4.2		
12.	{ 644, diseased 645, healthy	18	11	{ 20.4 (2) 35.6 (2)	{ 25.2 (2) 31.6 (2)	{ 25.7 (3) 31.6 (3)	{ 29.9 (3) 42.4 (2)	{ 35.6 (3) 55.0 (3)	{ 36.3 (3) 37.7 (3)	{ 19.2 $\pm$ 3.4 24.4 $\pm$ 3.4	{ 5.2 $\pm$ 4.8 10.2 $\pm$ 4.2		
13.	{ 646, diseased 647, healthy	13	8	{ 17.3 (2) 29.4 (2)	{ 25.2 (2) 31.6 (2)	{ 25.7 (3) 31.6 (3)	{ 29.9 (3) 42.4 (2)	{ 35.6 (3) 55.0 (3)	{ 36.3 (3) 37.7 (3)	{ 19.2 $\pm$ 3.4 24.4 $\pm$ 3.4	{ 5.2 $\pm$ 4.8 10.2 $\pm$ 4.2		
14.	{ 1007, diseased 1007, healthy	16	6	{ 29.4 (2) 35.6 (2)	{ 25.2 (2) 31.6 (2)	{ 25.7 (3) 31.6 (3)	{ 29.9 (3) 42.4 (2)	{ 35.6 (3) 55.0 (3)	{ 36.3 (3) 37.7 (3)	{ 19.2 $\pm$ 3.4 24.4 $\pm$ 3.4	{ 5.2 $\pm$ 4.8 10.2 $\pm$ 4.2		

<sup>1</sup> Description in text, p. 181. Numerals in parentheses indicate symptom intensity of attack by *C. lindemuthianum* as follows: 0=very slight lesions present on the veins of lower face of leaflet; 1=necrotic lesions plainly visible on veins of lower surface of leaflet; 2=necrotic lesions on most veins of upper and lower surfaces of leaflet; 3=necrotic lesions present in lamina adjacent to infected veins and slight loss of chlorophyll; 4=advanced stage of 3, with assumed loss of chlorophyll.

<sup>2</sup> Data taken between 8:00 a. m. and 12:30 p. m.

<sup>3</sup> Data taken between 12:30 p. m. and 5:00 p. m.



tion is found; this figure is highly significant. *Colletotrichum lindemuthianum*, therefore, reduced the assimilation of bean leaflets, as measured by the carbon dioxide utilized per unit area in unit time, by a significant amount.

No two inoculated leaflets showed the same response to fungus infection. In table 3, the consecutive stages in the development of symptoms on each leaflet are represented by the numerals 0, 1, 2, 3, and 4 placed in parentheses beside the assimilation reading. The presence of necrotic areas in the laminae of diseased leaflets did not reduce the assimilation rate as much as was expected from the symptoms observed. No measurements of necrosed areas per leaflet were made in the present investigation. A method is needed whereby daily measurement of diseased and healthy areas on a diseased leaf can be rapidly and accurately obtained without injury to the plant organ. Degrees of yellowing from loss of chlorophyll often characterized leaflets possessing necrosed areas. These symptoms may conceivably cause a reduction in the ability of cells to utilize carbon dioxide, but at present it is not known to what extent chlorophyll may be reduced in a cell before there is an apparent reduction in the rate of photosynthesis.

#### RESPIRATION

Data on respiration were obtained from most of the leaflets listed in table 3, and are presented in table 4 as mean respiration rates per 5-day period. The mean of all readings per leaflet during the course of the observations is given in table 5. Each mean is the average of 2 to 4 individual 4-hour readings. The periods in table 4 include the same time intervals indicated in table 3.

TABLE 4.—Mean respiration rates of pairs of opposite bean leaflets at four periods of maturity, when one member was healthy and the other was infected with *Colletotrichum lindemuthianum*, expressed as milligrams of carbon dioxide per 100 cm.<sup>2</sup> of leaflet surface per 4-hour determination

[Each mean is the average of 2 to 4 individual observations]

Pair of leaflets No.	Leaflet No. and condition	Mean respiration rates of leaflets at indicated period of maturity <sup>1</sup>				Mean of all observa- tions±S. F.	Differ- ence±S. E.
		Period A	Period B	Period C	Period D		
		Milli- grams	Milli- grams	Milli- grams	Milli- grams	Milligrams	Milligrams
1	{14, diseased	7.2 (1)	9.7 (1)			8.7±3.0	3.1±4.3
	{24, healthy	16.4	8.4			11.8±3.0	
2	{36, diseased	14.6 (1)	13.0 (2)			13.8±3.3	5.1±4.6
	{46, healthy	8.1	9.3			8.7±3.3	
3	{78, diseased	9.7 (0)	19.4 (1)			13.6±3.6	1.0±5.1
	{88, healthy	9.4	17.5			12.6±3.6	
4	{98, diseased	15.3 (1)	8.4 (1)			11.9±3.3	2.3±4.6
	{108, healthy	14.5	13.8			14.2±3.3	
5	{600, diseased	9.7 (1)	4.1 (3)	1.6 (4)		6.4±2.5	2.7±3.6
	{601, healthy	5.1	2.7	1.9		3.7±2.5	
6	{612, diseased	9.1 (1)	11.8 (2)	4.6 (3)	0.2 (4)	7.0±2.4	8.4±3.4
	{613, healthy	15.2	26.0	12.4	4.1	15.4±2.4	
7	{614, diseased	18.1 (1)	23.4 (2)	10.7 (3)		18.0±2.5	12.5±3.6
	{615, healthy	1.4	4.9	8.7		5.5±2.5	
8	{620, diseased	19.8 (1)	6.1 (2)	22.5 (2)		12.4±3.0	7.0±4.3
	{621, healthy	9.7	2.3	9.5		5.4±3.0	
9	{622, diseased	12.8 (0)	12.8 (1)	34.5 (1)		17.2±3.5	4.8±5.0
	{623, healthy	13.4	15.9	51.9		22.0±3.5	
10	{634, diseased			7.9 (3)	12.7 (3)	11.5±4.0	8.8±5.7
	{635, healthy			2.0	2.9	2.7±4.0	
11	{636, diseased	12.3 (1)	7.0 (2)	5.3 (2)		8.4±3.6	2.1±5.1
	{637, healthy	6.7	33.0	3.1		10.5±3.6	
12	{644, diseased			13.2 (3)	15.7 (3)	14.4±4.0	1.2±5.7
	{645, healthy			15.1	11.4	13.2±4.0	
13	{646, diseased		7.8 (2)	8.7 (2)	20.6 (2)	13.3±3.6	2.4±5.0
	{647, healthy		18.6	11.8	18.3	15.7±3.6	

<sup>1</sup> Description in text, p. 181. See also footnote 1, table 3.

Two pairs of opposite leaflets, namely, 612 and 613, and 614 and 615, showed significant differences between the respiration of the healthy member and that of the diseased member. The respiration of leaflet 612 (diseased) was less than that of leaflet 613, while the respiration of 614 (diseased) was greater than that of 615. There was no significant difference in respiration between healthy and diseased leaflets for any of the remaining 11 pairs of opposite leaflets.

Relative respiration rates of healthy and diseased bean leaflets at different stages of maturity are summarized in table 5. Diseased leaflets respired approximately 10 percent more carbon dioxide than healthy leaflets, but this difference is not statistically significant. For the plant material studied, it is concluded that infection with *Colletotrichum lindemuthianum* did not materially affect respiration. This somewhat unexpected result may be explained by assuming that necrosed areas respire less, and nonnecrosed areas, such as the margins of diseased spots, respire more than healthy tissues, and under the existing conditions these two opposite phenomena counterbalanced each other.

TABLE 5.—Mean respiration rates of all healthy and all diseased leaflets for which data are shown in table 4

Maturity of leaflets	Mean ( $\pm$ S. E.) respiration rate of—		
	Diseased leaflets	Healthy leaflets	Difference
	<i>Milligrams</i>	<i>Milligrams</i>	<i>Milligrams</i>
Period A	12.4 $\pm$ 1.5	9.7 $\pm$ 1.5	-2.7 $\pm$ 2.1
Period B	11.6 $\pm$ 1.5	11.2 $\pm$ 1.5	-.4 $\pm$ 2.1
Period C	9.9 $\pm$ 2.0	11.3 $\pm$ 1.9	1.4 $\pm$ 2.8
Period D	12.3 $\pm$ 2.7	8.5 $\pm$ 2.7	-3.8 $\pm$ 3.8
Average	11.6 $\pm$ .8	10.4 $\pm$ .8	-1.2 $\pm$ 1.2

#### PHYSIOLOGY OF PAIRS OF DISEASED LEAFLETS

The disease selected for study on pairs of diseased leaflets was mildew, caused by *Erysiphe polygoni* DC., which appeared as tiny spots on the upper surfaces of a number of full-grown bean leaflets on June 10, 1934. It is not known when inoculation occurred or how many spores produced infection per leaflet.

#### APPARENT PHOTOSYNTHESIS

Two observations per day were obtained on apparent photosynthesis. These data, together with the number of mildewed spots per leaflet and the color changes of each leaflet due to the action of the pathogen, are given in table 6.

The presence of mildewed areas on a bean leaflet did not materially reduce assimilation unless yellowing was also present; excessive yellowing was accompanied by a pronounced reduction in assimilation as compared with the assimilation of healthy leaflets (table 1). Abscission of leaflets outwardly little affected by *Erysiphe polygoni*, was not uncommon; this phase of the disease is probably due to toxic substances produced by the fungus and not to withdrawal of food or water loss. It is of interest to note that the assimilation of leaflet 12 closely paralleled that of the companion leaflet 22, though the latter showed twice

TABLE 6.—Daily assimilation and respiration rates of pairs of opposite bean leaflets infected with *Erysiphe polygoni*, expressed as milligrams of carbon dioxide per 100 cm.<sup>2</sup> of leaflet surface per 4-hour determination

[Each figure denoting assimilation is the mean of two determinations]

Pair of leaflets No.	Leaflet No.	Mildew lesions	Assimilation and respiration rates of leaves on—										Color and condition of leaflets			
			June 24		June 25		June 26		June 27		June 28	July 3			July 4	July 9
			Assimilation	Respiration	Assimilation	Respiration	Assimilation	Respiration	Assimilation	Respiration	Assimilation	Assimilation			Assimilation	Assimilation
1	{ 12 22	Number 21 42	Milli-grams 38.6	Milli-grams 3.5	Milli-grams 37.4	Milli-grams 6.1	Milli-grams 37.1	Milli-grams 16.9	Milli-grams 30.1	Milli-grams 7.7	Milli-grams 21.8	Milli-grams 39.4	Milli-grams 37.8	Milli-grams 31.0	Dark green from June 24 to July 3, thereafter pale green. Dark green from June 24 to June 28; thereafter pale green. Pale green June 25; pronounced yellowing thereafter. Slight yellowing June 25; pronounced yellowing thereafter after June 27; abscission June 28. Yellowed. Pronounced yellowing. Pale green June 27; abscission June 28. Yellowed. Dark green. Do.	
			41.8	6.4	42.6	.8	48.8	5.6	35.5	12.5	29.7	32.2	33.5	21.6		
					12.2		20.8	9.2	8.6	10.6	11.1					
2	{ 13 23	4 6			27.2		20.3	9.2	25.5	2.4	8.8					
3	{ 34 44	>7 >7					4.8	8.6	19.7	12.0						
4	{ 95 105	>5 >5	4.5 5.6	12.0 16.8	31.7 4.6	4.0 .5	10.0 25.2 11.4	7.0 4.7 6.6	30.6	23.0	6.8					
5	{ 96 106	>5 >7							7.2	21.3						
6	{ 97 107	8 10			48.2 29.6	15.1 10.6	43.5 32.2		-1.5 51.9 37.6	11.0	-2.4					

as many fungous lesions as leaflet 12. The assimilation of these two leaflets per unit area resembles that of healthy leaflets. Outward symptoms did not always give an accurate indication of the effect of the fungus on assimilation; for example, leaflet 44 was considered to be the more diseased of the pair 34 and 44, but from the assimilation data leaflet 34 apparently suffered the greater internal damage.

#### RESPIRATION

Data on the respiration of the mildew-affected leaflets are shown in table 6. The average respiration per 4-hour determination per leaflet is  $10.6 \pm 1.5$  mg. of carbon dioxide per 100 cm.<sup>2</sup> Healthy leaflets respired  $10.4 \pm .8$  mg. of carbon dioxide per 100 cm.<sup>2</sup> in a similar length of time (table 5). While the latter figure is not strictly comparable with the present data, the two results are so similar as to suggest that in the present experiments *Erysiphe polygoni* had little influence on respiration.

#### APPARENT PHOTOSYNTHESIS OF BEAN LEAFLETS INJURED BY FEEDING OF THRIPS

Measurements of assimilation were made on mature bean leaflets showing symptoms associated with the feeding of *Heliothrips haemorrhoidalis* (Bouché), the common greenhouse thrips. All stages of injury were present on the leaflets studied, from initial surface rasping to loss of chlorophyll in varying degrees. Plants bearing affected leaflets were fumigated with nicotine before determinations were started. Symptoms continued to develop after the thrips had been removed; the loss of chlorophyll increased until definite yellowing could be detected, and occasionally abscission of leaflets occurred. Abscised leaflets were generally dark green and outwardly fully turgid when leaflet fall occurred. This latent action of the feeding of the thrips is thought to be due to some toxic substance introduced by the insect. The fumigation with nicotine is not judged to have exerted any detrimental effect; healthy leaflets on the same plants as the thrips-injured leaflets showed no loss of chlorophyll or premature abscission. No data were obtained on the number of thrips that had fed on the leaflets or the length of the feeding prior to the start of physiological readings.

Photosynthesis readings for five pairs of leaflets, taken after feeding by thrips, are given in table 7; notes on the intensity of insect injury are included.

In figure 2, data obtained from an additional pair of leaflets (32 and 42) are presented. This pair of leaflets in a healthy condition was studied from May 23 to May 29, 1934. The rate of assimilation of the two leaflets was very similar and the difference between their mean assimilation rates is not significant (table 1). The plant bearing the leaflets was transferred to the greenhouse on May 30, and on June 12 both leaflets showed evidences of the feeding of thrips. The plant was fumigated with nicotine and returned to the assimilation chamber, where further determinations were made at intervals until June 23.

The data presented in table 7 and in figure 2 show that an appreciable reduction (43 to 80 percent) in the assimilation rate followed the feeding by thrips. However, this lowering of the assimilation rate undoubtedly includes reductions due to natural senility, for these leaflets were older than any of the other leaflets studied.

Reductions in assimilation were proportional to the apparent severity of the insect injury.

TABLE 7.—Daily assimilation rates of pairs of opposite bean leaflets that had been injured by the feeding of *Heliothrips haemorrhoidalis*, expressed as milligrams of carbon dioxide per 100 cm.<sup>2</sup> of leaflet surface per 4-hour determination, with notes on the severity of insect injury, 1934

Pair of leaflets No.	Leaflet No.	Type of injury <sup>1</sup> and daily assimilation rate of leaflets on—							
		May 19		May 20		May 21		May 22	
		Type of injury	Assimilation rate	Type of injury	Assimilation rate	Type of injury	Assimilation rate	Type of injury	Assimilation rate
			Milli-grams		Milli-grams		Milli-grams		Milli-grams
1	31	R	45.2	R	41.7	R	39.5	R	35.1
	41	R	39.1	R	54.1	R	41.5	R	67.8
	72			R	55.1	R	33.8	RR	22.9
2	82			R	21.8		28.0	R	29.8
	76	R	28.2	R	17.9	RR	23.3	RR	22.5
3	86	R	16.6	R	33.6	R	21.6	RR	3.1
	91	RR	9.2	RR	21.6				
4	101	R	20.9	R	45.4				
	92								
5	102							RRR	24.5
								RRR	9.4

<sup>1</sup> R=rasping of leaflet surface with little or no loss of chlorophyll; RR=rasping accompanied by slight yellowing; RRR=rasping accompanied by much yellowing.

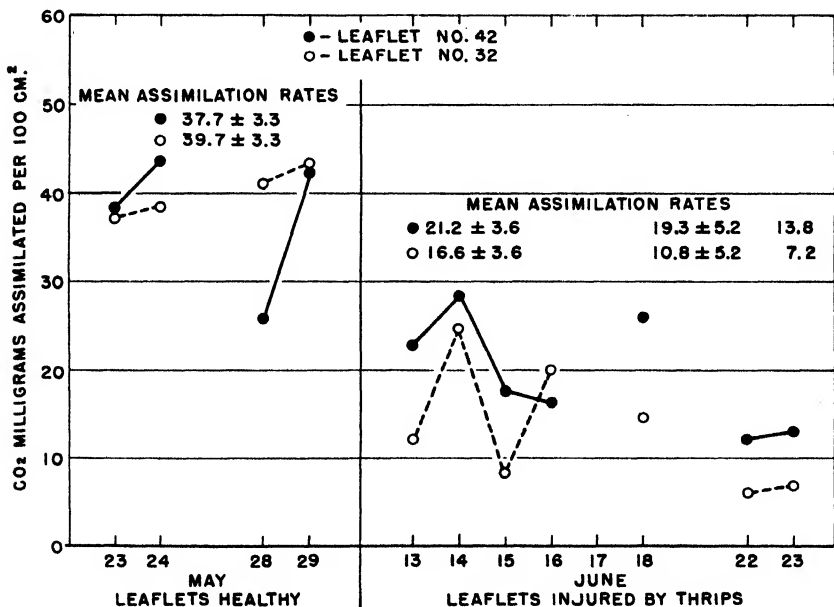


FIGURE 2.—Assimilation per 4-hour period of a pair of opposite bean leaflets (Nos. 32 and 42), both healthy, May 23-29, 1934; both injured by thrips June 13-23. Expressions of insect injury were as follows: Rasping with little loss of chlorophyll: Leaflet No. 32, June 13-15; leaflet No. 42, June 13-17. Slight yellowing: Leaflet No. 32, June 16-18; leaflet No. 42, June 18-21. Pronounced yellowing: Leaflet No. 32, June 19-23; leaflet No. 42, June 22-23.

## SUMMARY

Between the members of a pair of healthy and opposite bean leaflets, there are no statistically significant differences in (1) assimilation of carbon dioxide per unit area in unit time, or (2) growth, as measured by either increase in area or total area. Between different pairs of healthy leaflets on the same or on different plants, significant differences in assimilation are occasionally found. Differences greater than 15 percent are considered statistically significant.

The assimilation rate of healthy bean leaflets, even on a unit-area basis, increases with maturation of the leaflets. Studied for 15 consecutive days, with determinations started 13 to 15 days after seeds were planted, leaflets assimilate progressively greater and greater amounts of carbon dioxide. The maximum point in the assimilation curve in relation to age of the bean leaflet was not determined.

Leaflets infected with *Colletotrichum lindemuthianum* (Sacc. and Magn.) Bri. and Cav., assimilate 24 percent less carbon dioxide than healthy companion leaflets, and are restricted in growth by approximately 1.6 percent. Both of these differences are statistically significant. No statistically significant difference has been found between the respiration of diseased and healthy leaflets.

*Erysiphe polygoni* DC. does not reduce normal assimilation until yellowing, due to the action of the fungus, is produced. Diseased leaflets often abscise prematurely while still dark green and turgid. No significant difference has been found between the respiration of mildew-affected and healthy leaflets.

Feeding injuries by thrips (*Heliothrips haemorrhoidalis* (Bouché)) cause a loss of chlorophyll and yellowing and an appreciable reduction in the assimilation rate; the reduction is proportional to the apparent severity of the insect damage.

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## GROWTH AND FLOWERING OF SOME TAME AND WILD GRASSES IN RESPONSE TO DIFFERENT PHOTOPERIODS<sup>1</sup>

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### INTRODUCTION

In 1934 a paper appeared under the joint authorship of Evans and Allard (7)<sup>2</sup> embodying the results of a study of the growth and flowering behavior of 16 strains of timothy in response to different constant daily light periods, all but 4 strains being of American origin. The 4 not of American origin originated from seed obtained from northern Europe and were shown to attain the flowering condition at later dates than the American strains. It was indicated in this paper that the observed earliness or lateness of the various timothy strains was mainly due to inherent differences of response to the seasonal length of day following the awakening of growth in springtime.

Further discussions of the length-of-day behavior of timothy strains were embodied in papers by Evans, Allard, and McConkey in 1935 (8), and by Evans in 1939 (6).

Earliness of flowering appears to depend upon the fact that some varieties lay down the flower primordia in response to shorter day lengths than the later flowering varieties. The latter must await the coming of the longer days of summertime before the flower primordia, if initiated, can develop into a normal flower.

Varieties have been found that in the latitude of Washington, D. C., flowered very sparsely or not at all, even when the length of the midsummer day had attained its greatest value, nearly 14.9 hours. The English strain of timothy known as Harpenden, reported upon in the present paper, is of this type.

In view of the fact that this study of some of the more important tame grasses, begun by Evans and Allard, has been extended to include a number of other very important lawn and forage types, it has seemed advisable to present the behavior of these in the present paper.

It is well known that vast areas of the United States in the West are comprised of natural grasslands. It is also obvious that, in the great forest belts of the more humid regions, the woodland on the more arable soils everywhere has had to give way to an artificially established grassland, which comprises lawns and pastures and also meadows for the production of crops of hay in the dairy and stock-raising regions. In other words, the American system of agriculture to a great degree involves the cultivation of the various grasses for one purpose or another, and much of the success in grassland agriculture

<sup>1</sup> Received for publication October 22, 1940.

<sup>2</sup> Italic numbers in parentheses refer to Literature Cited, p. 227.



has come with the successful introduction of European types adapted originally to European fields and climate.

In the past the introduction of many exotic grasses has been largely an empirical procedure. With the discovery of the importance of the principle of the length-of-day responses of plants in 1920 by Garner and Allard (9), botanists have come to realize that the successful introduction of useful plants into new regions may depend largely upon their length-of-day behavior, as well as upon their temperature limitations and other factors. Obviously far northern plants are adapted to the long days and cool temperatures of high latitudes, and their flowering and fruiting, other conditions being favorable, can be expected in approximately similar temperatures and latitudes both north and south of the equator. Whether or not such plants can be grown under the shorter equatorial days depends as much upon the range of their temperature and day-length responses as upon any other factor. As a matter of fact, if high temperature is not unfavorable, length of day may often be the limiting factor, since temperatures low enough to interfere with growth do not ordinarily occur in equatorial regions.

In many plants, especially the herbaceous grasses, the character of the vegetative growth, as well as that of flowering and fruiting, may be profoundly altered by the length-of-day factor. Under one set of conditions the plants may produce flowering stems abundantly, with the expression of sexual reproduction dominant. Under another set of day-length conditions, sexual reproduction may be entirely or partially suppressed and vegetative growth accentuated. The dominance of this phase of growth expression leads to vigorous sod formation by stooling, stolon formation, and nodal branching.

These two categories of growth behavior to a certain extent may be antagonistic, since factors causing a strong dominance of one may retard or weaken the expression of the other. Since they represent continuities of a single life history, however, it is evident that certain borderline lengths of day may be favorable to a close interplay of both activities. In such instances, purely vegetative growth is more nearly in equilibrium with sexual reproductive expression. It is here that the largest growth form and the most vigorous fruiting may take place, favorable to high hay and forage yields.

In the case of the lawn grasses a greater degree of vegetative dominance is more naturally expected, for this leads to better mat or sod formation owing to a rapid lateral extension of the original plant, with little or none of its energies given over to flower-stem and seed formation.

A basic and thorough understanding of the ecological effects of seasonal length of day alone upon the life history of the grasses involves considerable study and analysis. The seasonal length of day in the middle latitudes is ever an inconstant condition. Increasing amplitudes of light prevail from spring to midsummer, and decreasing values from midsummer to autumn. The former condition favors sexual expression in the long-day grasses; the latter, in the short-day grasses. As has been indicated above, when sexual expression is attaining its maximum condition purely vegetative expression is suffering suppression, and the converse relation obtains when vegetative expression is at its maximum. This seasonal interplay of wide-

limit variations in length of day exercises very marked effects upon the development of vegetation in the higher temperate latitudes and very largely determines the life history of the grasses as we see their expression in American farm economics. In equatorial regions the seasonal swing becomes very narrow, and if there are day-length responses in the vegetation these must depend upon very small increments and decrements. It is evident that in the high Arctic latitudes, where constant daylight obtains throughout the growing season, the plants experience no seasonal changes in length of day, and such phases of development as they show must depend upon other factors than light-darkness ratios. A slight change in latitude near the critical length of day for the best expression of a particular phase of growth may thus profoundly modify the entire course of development of the particular species and militate against its usefulness for a given purpose. It must be understood, however, that length of day is but one of many factors operating at all latitudes, and that temperature, more especially, may greatly modify the developmental processes.

In the propagation of the grasses vegetatively, likewise, there may be decided advantages depending upon the suitable length-of-day factor. It would appear that, when vegetative dominance is at its maximum, rapid growth and extension of the clones is most favored. For this reason vegetative propagation is favored under those lengths of day that are less than the critical length for flowering or sexual reproduction. Vegetative propagation of the long-day grasses in the greenhouse in wintertime is dependent upon this principle, and the rapid lateral extension of the clumps under similar short photoperiods artificially produced in summertime is additional proof of its operation.

A proper and conservative recognition of the length-of-day factor, therefore, as affecting the expression of the grasses, may not only clarify the logic of much of the empirical success in the past, but make it possible to handle and to distribute newer introductions in a more scientific manner and therefore with greater success.

## MATERIAL AND METHODS

The investigations reported upon were carried out at Arlington, Va., with the equipment of the Division of Tobacco Investigations located there.

The present paper deals with 13 species and varieties or strains of grasses, including the following:

- Canada bluegrass (*Poa compressa* L.), introduced from the Old World (table 2).
- Kentucky bluegrass (*Poa pratensis* L.), introduced from the Old World (table 3).
- Orchard grass (*Dactylis glomerata* L.), introduced from the Old World (table 4).
- Wirestem muhly (*Muhlenbergia mexicana* (L.) Trin.), native of North America (table 5).
- Nimblewill (*Muhlenbergia schreberi* Gmel.), native of North America (table 6).
- Reed canary grass (*Phalaris arundinacea* L.), introduced from the Old World (table 7).
- Smooth brome grass (*Bromus inermis* Leyss.), introduced from the Old World (table 8).
- Timothy (*Phleum pratense* L.), introduced from the Old World (tables 9 and 10).
- Bulbous bluegrass (*Poa bulbosa* L.), introduced from Europe (table 12).
- Creeping bent (*Agrostis palustris* Huds.), native of America and Europe (table 13).
- Indian grass (*Sorghastrum nutans* (L.) Nash), native of North America (table 14).
- Bottlebrush (*Hystrix patula* Moench), native of North America (table 15).
- Eastern gamagrass (*Tripsacum dactyloides* (L.) L.), native of North America (table 16).

Most of these grasses are among our most valuable species, having a high degree of usefulness for one purpose or another in American grassland agriculture.

In the present paper, three strains of Kentucky bluegrass are being reported upon, one early, one medium-late, and one a late strain. Five strains of timothy have been tested, including one of Harpenden, England, origin; one a Russian (Moscow) strain; one designated as Welsh S. 50, a pasture variety; and the American varieties Huron (1937) and Marietta (11901).

Two strains of creeping bent (*Agrostis palustris*) that have come into use for lawns and golf greens have been studied. These are known as Metropolitan bent and Washington bent.

The majority of the grasses reported upon in this paper are Old World natives that have long been established in North America. Many of these species have a very wide distribution, and the results of the present study would indicate the importance of day-length studies for grasses in general.

Indian grass (*Sorghastrum*) and bottlebrush (*Hystrix*) are familiar wild North American species, the former being a common constituent of the tall-grass prairies of the Middle West and a normal grass component of the old-field successions of the East. Bottlebrush is confined almost strictly to deciduous woodlands. It is a beautiful and graceful grass, but of no present economic value. Oftentimes it occurs in patches of considerable size in the heavy woodlands of the mountain areas; and on some of the shale barrens of the Massanutten ridges in Virginia it appears to have a place in certain successions, forming a dense ground cover where duff and humus have accumulated.

The three timothy strains, Harpenden, Russian (Moscow), and Welsh strain S. 50, were received as large clumps from Canada, through the efforts of Prof. O. McConkey, of Agricultural College, Guelph, Ontario. These clumps were divided March 18, 1936, potted in 3-inch pots, and kept in a cool greenhouse with temperatures ranging from 50° to 55° F. On March 28, 1936, these pots were transferred to open coldframes to keep the plants dormant so far as possible. These plants made excellent growth, filling the small pots with leafage, and on March 30 they were transferred to 14-quart galvanized-iron buckets and to tests when about 2 inches in height.

The two timothy varieties, F. C. 3937 and F. C. 11901, were received as large clumps from Ohio, February 1937. The former was divided and potted in thumb pots February 23 and kept in the cool house until April 7, when the plants were transferred to pails and tests, the leafage then being 3 inches tall.

The clump of 11901, after being divided, was planted in 4-inch pots February 10 and kept in the cool house (50° to 55° F.) until March 4, when the plants were transferred to the coldframe out of doors. This material was finally placed in pails and transferred to the tests March 31, when the leafage was 6 inches tall.

All the remaining species, except the creeping bents, the Indian grass, and the bottlebrush, were received as large clumps from Ohio. These were divided and the plants placed in thumb pots or in 3-inch pots December 21, 1935.

The brome grass, Canada bluegrass, reed canary grass, and wirestem muhly were first potted in thumb pots December 21 and were kept in the cool greenhouse until January 30, 1936, when they were trans-

ferred to 3-inch pots. They were placed in the coldframe out of doors February 27, to remain until March 27, when they were transferred to 14-quart galvanized-iron buckets and to the tests beginning on that date.

The orchard grass and the three strains of bluegrass were first potted in 3-inch pots December 21, 1935, and remained in the cool greenhouse until March 16, 1936, when they were transferred to the galvanized-iron buckets and to the tests beginning March 27.

In one test *Poa bulbosa* was grown from bulbils formed normally in the inflorescence. These were sowed in flats in the cool greenhouse March 23, 1937, and germinated March 29; a clump of these plantlets was transplanted to buckets and placed in the tests April 23, when the plants were about 2 inches high.

In another test, clumps of overwintering *Poa bulbosa* were transplanted directly from the field to buckets March 25, 1937, and transferred to the tests March 29, when the shoots were only 1 inch high.

The Washington bent and the Metropolitan bent were transferred March 25, 1937, as small clumps, to buckets from the field at Arlington, Va., where they had passed the winter; the tests were begun on March 27. At that time the shoots were about 1 inch tall.

Material of *Muhlenbergia schreberi* was received from Ohio in February 1937 as large clumps, which were split up and potted to thumb pots February 23. They were later transferred to buckets and to the tests April 23, when the leafage was about 3 inches high.

*Sorghastrum nutans* and *Hystrix patula* were grown from seed obtained in the fall of 1937 from wild plants, the former from Little Cobbler Mountain and the latter from Bull Run Mountain, both in Fauquier County, Va. The seed was sown in flats in the coldframe December 16, 1937, which remained exposed to the normal cold of the wintertime until January 29, 1938, when the flats were brought into the cold greenhouse. Germination took place February 16 in the case of *Sorghastrum*, and February 4 in the case of *Hystrix*.

The *Sorghastrum* plants were placed singly in thumb pots March 23 and were transferred to buckets May 4, when 7 inches tall, three plants being grown in each bucket. The tests were begun on May 4.

The *Hystrix* plants were placed in thumb pots February 28 and transferred to pails May 3, when 4 inches tall, three plants being grown in each bucket. The tests were begun on May 3.

The material of the eastern gamagrass was obtained by dividing a large clump found near the Arlington Experiment Farm, Arlington, Va., and planting portions in buckets March 31, 1937. At this time shoots about 2 inches high were in evidence. The tests also were begun on that date.

It may be stated that with the exception of these grasses grown from seed, of which there were only two, *Hystrix* and *Sorghastrum*, all clumps of material represented clonal multiplication of selected strains. The plants were grown in 12- or 14-quart buckets with drainage holes in the bottom; one bucket was used for each test on a given treatment with the exception of the controls, represented by two buckets. The original clumps were taken from field material, and therefore experienced the normal outdoor conditions until divided and grown in the greenhouse in small pots, under temperatures of 50° to 55° F.

It is evident that all the tests within this range could not begin at the same time owing to seasonal differences in length of day; the 12-hour

day did not begin until near March 21, and days longer than that at later dates. For this reason the tests with the longer light intervals were begun when the natural length of day had reached the particular duration desired. Until that time the plants received the normally increasing daylight periods. The data in table 1, showing the constant daily light periods used and the date when the tests for each were begun, will make this clear.

TABLE 1.—*Length of the daily light periods used and dates when tests were begun*

Light period		Date tests were begun
Duration (hours)	Clock time	
10	6 a. m. to 4 p. m.	Mar. 27
12	6 a. m. to 6 p. m.	Do.
12.5	6 a. m. to 6:30 p. m.	Mar. 29
13	5 a. m. to 6 p. m.	Apr. 10
13.5	5 a. m. to 6:30 p. m.	Apr. 23
14	5 a. m. to 7 p. m.	May 6
14.5	5 a. m. to 9:30 p. m.	May 21

In all tests involving a range of 10 to 14.5 hours of light inclusive, natural daylight was used, darkness being secured by placing the plants in lightproof, ventilated houses each day. This was accomplished by keeping the buckets upon movable trucks mounted upon tracks. These trucks were moved in and out of the darkened houses, according to the schedules outlined above, to obtain each light period.

Since the constant light periods designated as 16 and 18 hours are in excess of the normal daylight periods of the Washington latitude, the use of supplemental electric light, from sunset on, was required to obtain these photoperiods. In these tests the added illumination was obtained from four 200-watt gas-filled tungsten lights with reflectors, mounted upon an iron frame that could be moved vertically to adjust the height of the lights, which were usually kept about 1 foot above the plants. These lights were arranged at the corners of a square the dimensions of which were 3 feet on a side from center to center of the lights. The intensity of the lights at this distance, as measured by a Weston illumination meter, model No. 1746, equipped with a Viscor filter to obtain as nearly as possible only visible radiation, amounted to about 300 to 400 footcandles.

Since the natural length of day is a variable, increasing to a maximum of about 14.9 hours at the summer solstice June 21, and decreasing at the same rate thereafter, constant light periods of 16 and 18 hours, respectively, required adjustments in the periods of artificial illumination after sunset to compensate for these seasonal increments and decrements of daylight throughout the growing season. This was done by the use of time switches, which were regulated at frequent intervals to maintain constant these periods of illumination.

One series of controls in buckets receiving the full seasonal length of day was maintained at the darkened house, while another similar series was maintained in the tests involving 16 and 18 hours of light, which were some distance away. The mean of these was taken as the representative figure in the tables. Plants of a number of the varieties that had overwintered in the field and also experienced the full length of day were also used for comparison.

The date of visible appearance of the first head from the boot, the date of pollination, the first noticeable evidence of yellowing or browning of the glumes or heads, and the date when about half the heads were straw-colored were noted for most of the varieties. The height of the tallest flowering stems was taken at the time pollen appeared. In some instances there was continued growth of the original flowering stem by branching, as in *Muhlenbergia mexicana* or *Bromus inermis*. In such cases later measurements sometimes showed greater lengths of stem under the column headed "Growth behavior" in the tables. Greater differences in this respect have often been shown where the natural day has been greatly extended by artificial light.

## EXPERIMENTAL RESULTS AND DISCUSSION

### CANADA BLUEGRASS

The behavior of Canada bluegrass (*Poa compressa*) (table 2) would indicate clearly that this species is a long-day type, since daylight periods of 10 hours to 13 hours inclusive were too short to allow flowering to take place. There was slight stem development, but no heads emerged in the tests of 12.5 and 13 hours. Although the plants receiving 13.5 hours of light flowered, there was some delay in the appearance of heads, showing that this length of day is near the upper limits of dominant vegetative expression. There was a particularly heavy growth of matted leafage where 10 and 12 hours of light were experienced. It is interesting to note that even where these plants experienced the full length of day at Washington, D. C., the bases of the stems were slightly decumbent, but with 16 and 18 hours of light, the stems were strictly erect. This is additional evidence that Canada bluegrass is an ecological form best adapted to very long days so far as flowering is concerned. As a matter of fact its range bears out this assumption, since it extends from Newfoundland and Alaska, southward to Georgia, Alabama, New Mexico, and California. At lower latitudes than this, this grass could be expected to reduce its flowering energies, or perhaps prove unable to flower, since lengths of day below 13.5 hours favor the dominance of vegetative expression. This grass appears to be well adapted to far northern regions (fig. 1).

TABLE 2.—Responses of Canada bluegrass to different constant daily light periods

Daily light period hours	Date when—				Tallest stem	Flower stems	Growth behavior
	First heads appeared	Pollen appeared	First glumes were straw-colored	Half of heads were straw-colored			
Hours					Inches	Number	
10	None	None	None	None	None	None	Heavy rosette growth; no stem elongation.
12	do	do	do	do	None	None	Do.
12.5	do	do	do	do	(1)	None	Slight stem elongation; very decumbent; no heads.
13	do	do	do	do	(1)	None	Do.
13.5	June 1	June 16	June 22	July 13	16	42	Somewhat decumbent at base.
14	May 25	June 11	June 19	do	15	81	Do.
14.5	May 20	June 8	do	July 6	17	147	Do.
16	May 12	June 1	June 11	June 18	24	138	Stems erect.
18	do	do	do	June 16	25	163	Do.
(2)	May 25	June 13	July 1	do	18	156	Stems somewhat decumbent at base.
(3)	May 5	June 12	June 30	July 17	14	64	Do.

<sup>1</sup> Slight. <sup>2</sup> Full length of day; plants in buckets.

<sup>3</sup> Full length of day; plants in the field.

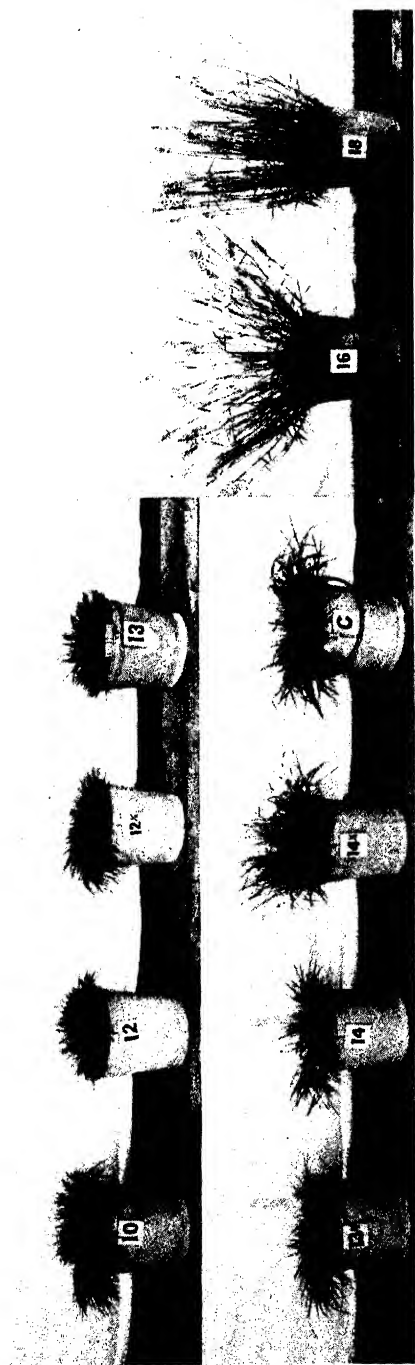


FIGURE 1.—Responses of Canada bluegrass (*Poa compressa*) to various constant daily photoperiods (hours) indicated. Control (C) received full length of day. There was no flowering until 13.5 hours of light each day were experienced, only a few stems being produced on the 12- and 12.5-hour photoperiods. Dates of flowering with various photoperiods were as follows: With 13.5 hours, plants flowered June 16; 14 hours, June 11; 14.5 hours, June 8; full length of day (C), June 13; 16 hours, June 1; 18 hours, June 1. On the shorter lengths of day the stems were very decumbent, becoming strictly erect on the longer days. Canada bluegrass behaves as a typical long-day type of plant. Photographed May 29, 1936.

## KENTUCKY BLUEGRASS

Three strains of Kentucky bluegrass (*Poa pratensis*) have been grown, one an early strain, one medium-late, and one a late strain (table 3).

From the dates of heading and the appearance of open florets liberating pollen, the early strain shows an advance of 3 to 4 weeks or more in heading over the medium-late and the late strains, the time of flowering also being earlier. The stems of the early strain were slightly decumbent in response to 10 hours of daylight, but were erect under all longer light periods (fig. 2).

TABLE 3.—Responses of three strains of Kentucky bluegrass to different constant daily light periods

Daily light period	Date when				Tallest stem	Flower stems	Growth behavior
	First heads appeared	Pollen appeared	First plumes were straw-colored	Half of heads were straw-colored			
<i>Hours</i>					<i>Inches</i>	<i>Number</i>	
10	Mar. 2.	Apr. 4	May 18.	May 26	17	26	Stems slightly decumbent.
12	do	Apr. 14	May 20	May 20.	15.5	22	Stems erect.
12.5	do	do	do	May 27	17	17	Do.
13	do	Apr. 29	do	May 29	20	16	Do.
13.5	do	Apr. 11	May 22	do	15.5	25	Do.
14	do	do	do	do	13.5	16	Do.
14.5	do	Apr. 27	May 19	June 5	16	13	Do.
16	do	Apr. 10	do	May 28	17	17	Do.
18	do	Apr. 16	May 18	May 27	15	17	Do.
(1)	do	do	do	do	17	18	Do.
(2)	do	Apr. 25	May 20	do	14.5	13	Do.

MEDIUM-LATE STRAIN							
10	Mar. 25	Apr. 27	May 20	May 29	13	18	Stems very decumbent, lying almost flat upon the ground.
12	do	May 2	May 22	May 27	14	35	Do.
12.5	do	May 6	May 21	May 29	17	27	Stems less decumbent than in 10- and 12-hour tests.
13	do	Apr. 29	May 20	do	20	25	Stems more erect than for the 12.5-hour test.
13.5	do	May 4	do	May 28	20	20	Stems declined about as in the 12.5-hour test.
14	do	May 6	May 19	May 29	20	21	Stems more erect than for the 13.5-hour test.
14.5	do	May 7	May 21	June 4	13	22	Stems almost erect; only very slightly declined.
16	do	May 4	May 22	May 28	15	17	Stems strictly erect.
18	do	May 7	do	May 27	18.5	26	Do.
(1)	do	Apr. 28	May 18	May 28	15.5	23.5	Do.
(2)	do	May 4	May 25	May 29	17	13	Do.

LATE STRAIN							
10	May 2	May 11	May 27	June 6	9	10	Stems somewhat declined.
12	Apr. 13	May 8	May 26	June 3	10	26	Stems nearly erect.
12.5	Apr. 15	May 4	May 21	June 4	12	19	Stems strictly erect.
13	do	May 8	May 23	June 3	11	30	Do.
13.5	Apr. 13	May 4	May 22	do	18	16	Do.
14	Apr. 15	May 7	May 23	do	15	22	Do.
14.5	Apr. 14	May 8	May 19	June 4	17	19	Do.
16	Apr. 20	May 11	May 25	June 11	18	15	Do.
18	Apr. 15	May 7	do	June 1	17	16	Do.
(1)	Apr. 13	May 5	May 19	June 3	16	23	Do.
(2)	Mar. 28	May 11	May 25	May 27	12	8	Do.

<sup>1</sup> Full length of day; plants in buckets.

<sup>2</sup> Full length of day; plants in the field.





FIGURE 2.—Responses of early strain of Kentucky bluegrass (*Poa pratensis*) to various constant daily photoperiods (hours) indicated. Control (C) received full length of day. All the plants flowered readily. Dates of flowering with various photoperiods were as follows: With 10 hours, plants flowered April 4; 12 hours, April 14; 12.5 hours, April 29; 13.5 hours, April 29; 14 hours, April 14; 14.5 hours, April 27; full length of day (C) April 16; 16 hours, April 10; 18 hours, April 16. This early strain shows an indeterminate length-of-day behavior, the stems becoming more erect on the longer days. Photographed May 29, 1936.

The medium-late strain and the late strain of Kentucky bluegrass are less sharply defined in their responses than the early strain, yet in time of heading the medium-late strain is about 3 weeks earlier. This strain is rather distinctive, also, in that it shows very decumbent stems lying almost flat upon the ground when the daily light periods were maintained at 10 and 12 hours. The flowering stems of this strain did not become strictly erect until the full length of day and the 16- and 18-hour light periods were experienced.

The stems of the late strain of Kentucky bluegrass showed only a slightly declined growth in response to 10 hours of daylight, became nearly erect with 12 hours, and strictly erect with all longer light periods.

In the case of these three strains, the tendency toward the decumbent habit of growth does not appear to be associated entirely with length of day. The medium-late strain is perhaps inherently more of a prostrate type of grass than either the early or the late strain.

Kemp (12), of the Maryland Agricultural Experiment Station, has shown that the close grazing of pastures is an important biotic factor in the production of low and more or less prostrate types of grass and white clover. In such pastures he found the surviving types of bluegrass and orchard grass to be short and procumbent, as compared with the taller strains found in hayfields. In other words, a rigid natural selection has taken place, leaving only the smaller, more prostrate types to survive because they were better fitted to escape destructive close cropping.

While there may be a more or less inherent difference in the degree of expression of the prostrate habit of growth in the case of the three bluegrass strains here reported upon, the factor of length of day has been shown to modify greatly the expression of this growth form.

Kentucky bluegrass is of European origin, where the summer days are very long, and it is indicated that this grass is well adapted to the cooler northern regions, where long summer days prevail.

In the case of timothy strains, ranging by fairly uniform gradations from very early to very late in time of flowering, the stems become increasingly procumbent with increased lateness and required gradually longer daily light periods to attain erect growth.

Of the three strains of Kentucky bluegrass reported upon, the medium-late strain apparently has not followed this typical behavior of timothy, since it was more procumbent than the later strain. Further study of the various Kentucky bluegrass strains would be necessary to obtain a clear understanding of the differences of behavior between timothy and these bluegrasses.

#### ORCHARD GRASS

As the data indicate in table 4, orchard grass (*Dactylis glomerata*) is favored by the longer light periods and to that extent is a long-day type. Although weak flowering occurred in response to 12 hours of daylight, the stems were so declined as to lie practically flat upon the ground, a behavior quite exceptional as compared with the normal erect growth of this tussock-forming grass in the field.

This markedly declined habit of stem growth was also shown in response to the 12.5-, 13-, and 13.5-hour light periods, and the stems

did not become strictly erect until 14.5 hours of light had been experienced.

Ten hours of light gave rise to dense tussocks of leafage only.

It is interesting to note that the time of flowering and heading was but little affected through the series of constant daily light periods, thus indicating a rather abrupt change from the purely vegetative to the reproductive phase of growth occurring at some period between 10 and 12 hours of light.

The distribution of orchard grass extends into Newfoundland and Alaska on the north, ranging southward to near the southern limits of the United States. This range could be predicted upon the data presented in table 4 and figure 3.

TABLE 4.—Responses of orchard grass to different constant daily light periods

Daily light period	Date when—				Tallest stem	Flower stems	Growth behavior
	First heads appeared	Pollen appeared	First glumes were straw-colored	Half of heads were straw-colored			
Hours					Inches	Number	
10	None	None	None	None	None	None	Dense rosette of leaves; no stem elongation.
12	May 7	May 20	May 29	June 11	22	2	Two flowering stems only; so declined as to lie nearly flat upon the ground.
12.5	May 8	do	June 2	June 12	20	8	Stems so declined as to lie nearly flat upon the ground.
13	May 12	May 26	June 3	June 15	20	7	Do.
13.5	May 6	May 19	May 29	June 11	26	8	Stems somewhat more erect than at the 13-hour period yet strongly declined.
14	May 8	May 23	June 3	June 16	36	10	Stems almost erect.
14.5	May 6	May 21	June 4	do	35	12	Stems strictly erect.
16	do	May 15	May 28	June 3	34	34	Do.
18	May 9	May 25	do	do	28	22	Do.
(1)	May 8	May 21	June 1	June 15	35	16	Do.
(2)	May 7	May 23	June 2	June 18	31	16	Do.

<sup>1</sup> Full length of day; plants in buckets.

<sup>2</sup> Full length of day; plants in the field.

#### WIRESTEM MUHLY

The grass known as wirestem muhly (*Muhlenbergia mexicana*) shows nothing more distinctive in its day-length responses than that 10 and 12 hours of daylight each day cause very low growth, with the heads on the short stems appearing almost on the ground.

With increase of day length, the flowering stems become noticeably longer and strictly erect. By July 8 the longer photoperiods had produced stems 25 to 30 inches long, as compared with stems only 3.5 inches tall on the 10-hour day.

On the basis of its length-of-day responses and stem elongation, this grass is better adapted to the longer days than to extremely short days. However, there is probably an optimum temperature range to which the grass may respond, since in parts of the South it grows on the mountains rather than on the warmer lowlands (table 5).

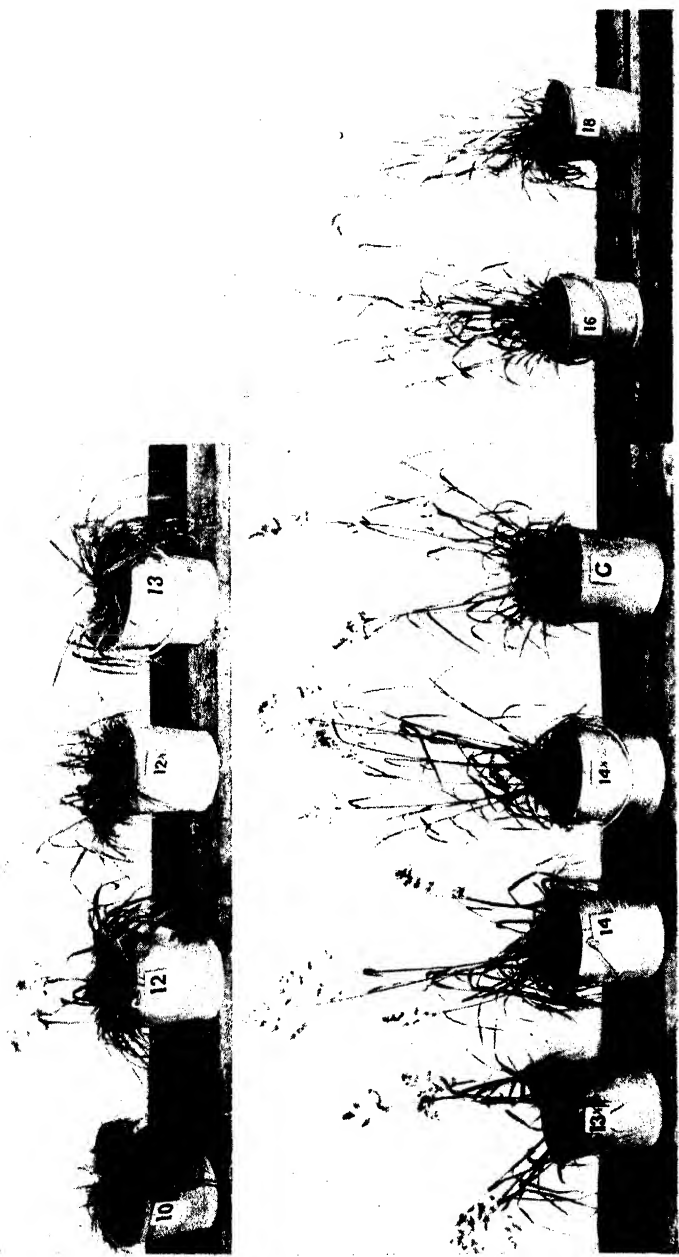


FIGURE 3.—Responses of orchard grass (*Dactylis glomerata*) to various constant daily photoperiods (hours) indicated. Control (C) received full length of day. Plants receiving 10 hours of light were unable to flower. Dates of flowering with various photoperiods were as follows: With 12 hours, plants flowered May 20 (few heads); 12.5 hours, May 26; 13 hours, May 26; 13.5 hours, May 18; 14 hours, May 23; 14.5 hours, May 21; full length of day (C), May 23; 16 hours, May 15; 18 hours, May 25. Orchard grass behaves as a typical long-day plant, with a very low critical photoperiod inhibiting flowering. Photographed May 29, 1936.

TABLE 5.—Responses of wirestem muhly to constant daily light periods

Daily light period	Date when—				Growth behavior
	First heads appeared	Pollen appeared	First glumes were straw-colored	Half of heads were straw-colored	
<i>Hours</i> 10	May 22	May 27	June 4	June 11	Growth very low; some heads appearing nearly on the ground, and could not be counted; tallest stem July 8 (3.5 in.) erect.
12	May 16	May 21	do	do	Do.
12.5	May 14	May 18	do	do	Same growth behavior as for 10-hour period; tallest stem July 8 (7 in.); erect.
13	do	May 21	do	do	Same growth behavior as for 10-hour period; tallest stem July 8 (14 in.); erect.
13.5	May 13	May 18	do	do	Same growth behavior as for 10-hour period; tallest stem July 8 (17.5 in.); erect.
14	do	May 19	do	do	Same growth behavior as for 10-hour period; tallest stem July 8 (18 in.); erect.
14.5	May 14	May 22	do	do	Same growth behavior as for 10-hour period; tallest stem July 8 (31.5 in.); erect.
16	May 9	May 21	June 11	June 18	Much new growth by nodal branching; no flowering from these; tallest stem July 8 (31 in.); strictly erect.
18	May 13	May 18	do	June 13	Same growth behavior as for 16-hour period; tallest stem July 8 (27 in.).
(1)	May 16	May 20	June 8	June 8	Tallest stem July 8 (22 in.).
(2)	May 15	May 18	do	do	Tallest stem July 8 (5 in.). <sup>3</sup>

<sup>1</sup> Full length of day; plants in buckets.<sup>2</sup> Full length of day; plants in the field.<sup>3</sup> Dry conditions in the field may have operated to check growth in these plants.

## NIMBLEWILL

The grass known as nimblewill (*Muhlenbergia schreberi*) is a native American species and, like *M. mexicana*, showed no very marked responses to the different photoperiods until artificial light was used to extend the length of day from sunset. With photoperiods of 16 or 18 hours, flowering was delayed a month or more as compared with those tests in which the natural day had been shortened by darkening. Only one or two heads appeared in these longer tests with artificial light, and a very characteristic prostrate growth of the stems was noticeable.

*Muhlenbergia schreberi* is a moisture-loving grass, and its range extends from the northern United States south to Florida, Texas, and eastern Mexico, and westward to Wisconsin and eastern Nebraska.

On the basis of its flowering responses under the various photoperiods presented in table 6, it is indicated that this grass is normally a native of the middle latitudes of the eastern half of the United States.

TABLE 6.—Responses of nimblewill to different constant daily light periods

Daily light period	Date when—				Tallest stem	Growth behavior
	First heads appeared	Pollen appeared	First glumes were straw-colored	Half of heads were straw-colored		
<i>Hours</i> 10	May 21	June 2	June 25	July 12	<i>Inches</i> 14	Very fine, compact green growth.
12	May 25	do	June 24	July 23	14	Do.
12.5	May 27	do	do	do	20	Full flower, Aug. 11.
13	do	June 21	do	July 12	20	New growth and flowering, Aug. 11.
13.5	do	June 2	June 30	July 15	21	Growing and flowering, Aug. 13.
14	May 26	June 9	July 3	do	17	Heading continued, flowering, Aug. 13.
14.5	do	do	July 8	July 21	16	Green and full flower, Aug. 16.
16	Aug. 15	Aug. 19	do	do	25	Two heads only; growth green but flat on the ground.
18	July 15	July 18	do	do	30	One head only; stems prostrate.
(1)	June 7	June 10	June 21	Aug. 3	17	No new growth, Aug. 19; old growth browning.

<sup>1</sup> Full length of day; plants in buckets.

## REED CANARY GRASS

Reed canary grass (*Phalaris arundinacea*) is quite plainly a long-day type of grass but is characterized by a rather low flowering limit as the days are decreased in length.

With 10 and 12 hours of light, the plants produced leafage only, and until 14 hours of light had been experienced few flowering stems arose and all were decumbent.

With 14.5 hours of light daily and with longer light periods, flower stems were produced more abundantly, these being erect or nearly so.

The time of flowering for the entire series of day lengths above 13 hours was very constant. The 12.5-hour test showed a delay of about 1 month, indicating that this was near the border line between the dominance of vegetative growth and the beginning of the flowering impulse. It is natural to believe that shorter light periods somewhere between the tests of 12.5 hours, where the plants flowered, though with delay, and 12 hours, where there were no indications of flowering, would have required still longer intervals to produce heads and flowering, until a point was reached where flowering ceased entirely.

The range of reed canary grass is mainly northern, extending to New Brunswick and Alaska. Southward its range appears to stop roughly on a line from North Carolina westward to California, reaching Oklahoma, New Mexico, and northern California.

This natural northern distribution is reflected in the response of the plants to the various increased daily light periods shown in the data of table 7 and in figure 4.

TABLE 7.—Responses of reed canary grass to different constant daily light periods

Daily light period	Date when ...				Tallest stem	Flower stems	Growth behavior
	First heads appeared	Pollen appeared	First glumes were straw-colored	Half of heads were straw-colored			
<i>Hours</i>					<i>Inches</i>	<i>Number</i>	
10	None	None	None	None	None	None	Leafage only; no flower stems.
12	do	do	do	do	do	do	Do.
12.5	June 20	June 25	July 6	do	28	1	Flowering stem somewhat decumbent.
13	May 23	June 1	do	do	27	1	Stem slightly decumbent.
13.5	May 25	May 28	June 29	July 6	21	(1)	Stem very decumbent, almost prostrate; one head May 25; shedding pollen June 16 at 20 in.
14	May 20	May 29	June 11	June 15	44	6	Stems very decumbent.
14.5	May 23	June 1	June 15	June 18	41	8	Stems mostly erect; only slightly declined.
16	May 19	May 26	May 28	June 11	46	8	Stems strictly erect; new stems forming July 8.
18	May 18	May 25	do	do	46	10	Do.
(2)	May 22	May 30	June 9	June 18	36	8	Stems mostly erect as in 14.5-hour day.
(3)	June 1	June 5	June 16	June 22	28	7	Stems rather noticeably declined.

<sup>1</sup> Several.

<sup>2</sup> Full length of day; plants in buckets.

<sup>3</sup> Full length of day; plants in the field.

## SMOOTH BROMEGRASS

The data for smooth brome grass (*Bromus inermis*) presented in table 10 indicate that we are dealing with a long-day type of plant.

In those tests involving the shorter light periods of 10, 12, 12.5, and 13 hours, dense leafage only was produced, or if stems formed they

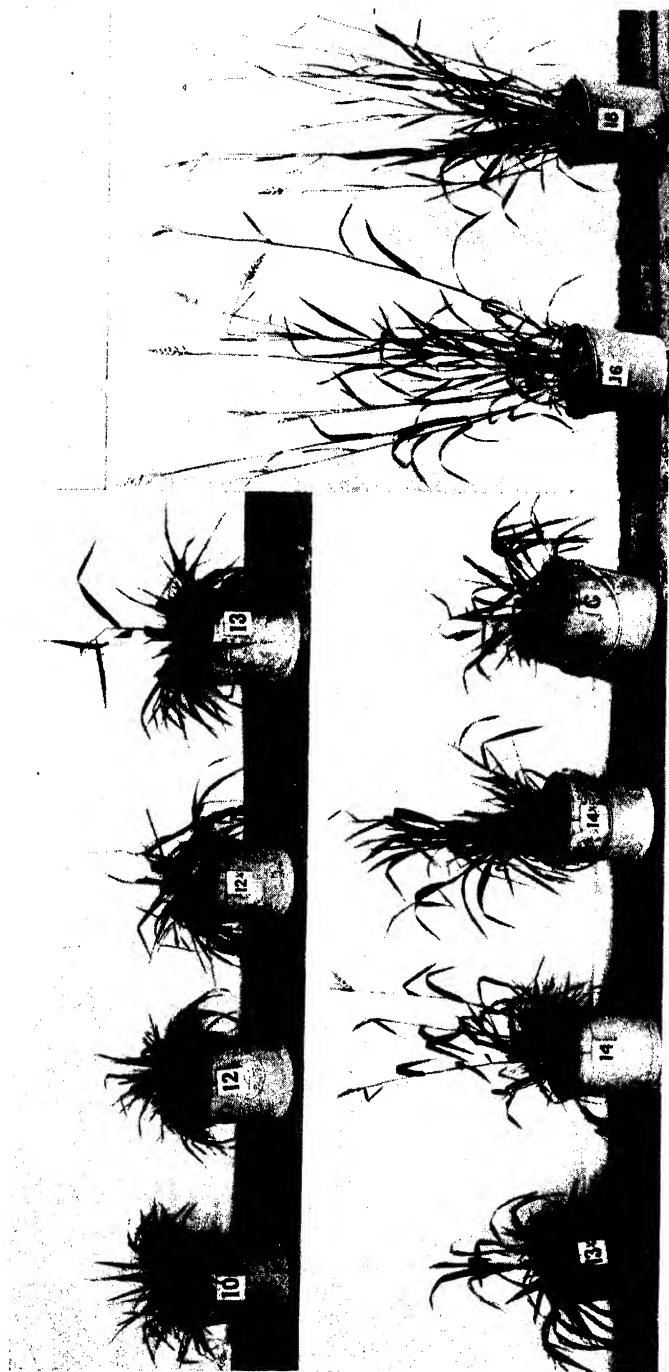


FIGURE 4.—Responses of reed canary grass (*Phalaris arundinacea*) to various constant daily photoperiods (hours) indicated. Control (C) received full length of day. Plants receiving 10- and 12-hour photoperiods never developed stems. Dates of flowering with various photoperiods were as follows: With 12.5 hours, plants flowered June 25; 13 hours, June 1; 13.5 hours, May 28; 14 hours, May 29; 14.5 hours, June 1; full length of day (C), June 3; 16 hours, May 26; 18 hours, May 25. This species is a typical long-day plant in its responses, flowering and producing erect stems as the days are lengthened. Photographed May 29, 1936.

were few in number and so decumbent as to lie flat upon the ground.

With increase in the length of the daily periods of illumination, the number of flower stems increased, their length was augmented, and they became nearly or quite erect.

TABLE 8.—*Response of smooth brome grass to different constant daily light periods*

Daily light period	Date when—				Tallest stem	Flower stems	Growth behavior <sup>1</sup>
	First heads appeared	Pollen appeared	First glumes were straw-colored	Half of heads were straw-colored			
<i>Hours</i>					<i>Inches</i>	<i>Number</i>	
10	None	None	None	None	None	None	Dense leafage only; no stems.
12	do	do	do	do	None	None	Do.
12.5	May 14	do	do	do	1	1	Mostly leafage; one stem, but head never emerged from sheath; stem very decumbent, flat upon ground. Single flower stem flat upon ground; mostly leafage.
13	May 6	May 23	do	June 22	9	1	
13.5	May 23	June 12	June 23	July 6	9	21	} Stems very much declined.
14	do	June 6	do	July 1	15	14	
14.5	May 26	June 11	June 29	July 6	18	21	Stems somewhat declined.
16	May 6	May 22	June 11	June 18	18	40	Stems more erect than at 14-hour period.
18	do	May 23	do	June 16	18	40	Stems erect; many new shoots evident June 2; 42 inches high.
(4)	May 27	June 12	June 18	July 4	19	29	Stems erect; many new shoots evident June 2; 33 inches high.
(5)	May 9	June 1	July 18	July 10	10	40	Stems somewhat decumbent at base. Do.

<sup>1</sup> When these measurements do not agree with those shown in the preceding column, this is due to the fact that the former measurements were made at a later date, during which interval increase in height had taken place.

<sup>2</sup> Early.

<sup>3</sup> Late.

<sup>4</sup> Full length of day; plants in buckets.

<sup>5</sup> Full length of day; plants in the field.

This grass is cultivated successfully as a hay and pasture grass in the more northern States, from Michigan and Kansas to Washington and Oregon. It appears best adapted to the northern half of the United States, and is escaping rather freely in this area. Its length-of-day responses as revealed in the text appear to afford confirmatory evidence of this tendency toward a more northern distribution (fig. 5).

#### TIMOTHY

It would appear from the work of the British investigators Gregor (10) and Gregor and Sansome (11) and from that of Witte of Sweden (14), that the timothy populations of the cultivated species *Phleum pratense*, as commonly understood, include a number of strains or types differing in growth form and other characters.

In their analysis of these forms, Gregor and Sansome designated the "American" and European cultivated types as group 1. These workers recognized a second group, the "wild," which they called group 2. Both groups have more or less striking growth form and behavior characteristics in the field.

The basic (gametic) chromosome number in the genus *Phleum* appears to be 7. The members of group 1, as discussed by Gregor and Sansome, including the cultivated or hay grasses, are distinguished by having 42 somatic (6 *n*) chromosomes, while the forms of group 2 are diploid, having 14 somatic (2 *n*) chromosomes.



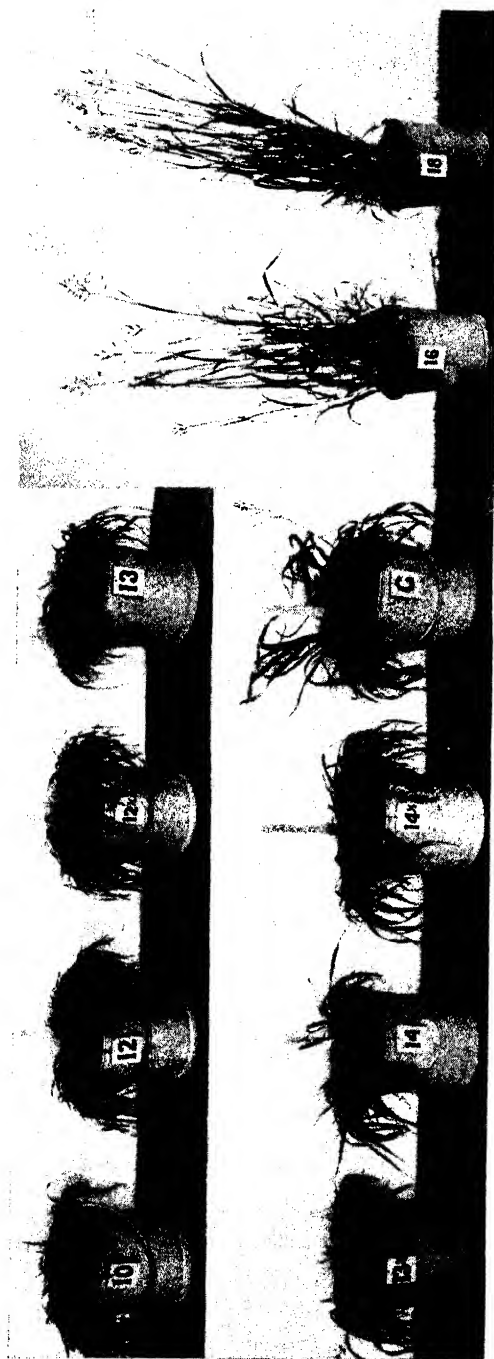


FIGURE 5.—Responses of bromegrass (*Bromus inermis*) to various constant daily photoperiods (hours) indicated. Control (C) received full length of day. Ten- and twelve-hour plants produced only leafage; 12.5-hour plants produced one decumbent head but did not flower. Dates of flowering with other photoperiods were as follows: With 13 hours, plants flowered May 23 (on one stem only); 13.5 hours, June 12; 14 hours, June 6; 14.5 hours, June 11; full length of day (C), June 8; 16 hours, May 22; 18 hours, May 23. This grass shows the behavior of a long-day plant. Photographed May 29, 1936.

It would appear that the earlier results presented by Evans and Allard (7) in 1934 were secured entirely with group 1, the hay varieties.

#### AMERICAN STRAINS

The length-of-day responses of the two timothy strains F. C. 3937 (Huron) and F. C. 11901 (Marietta) are shown in table 9. Both

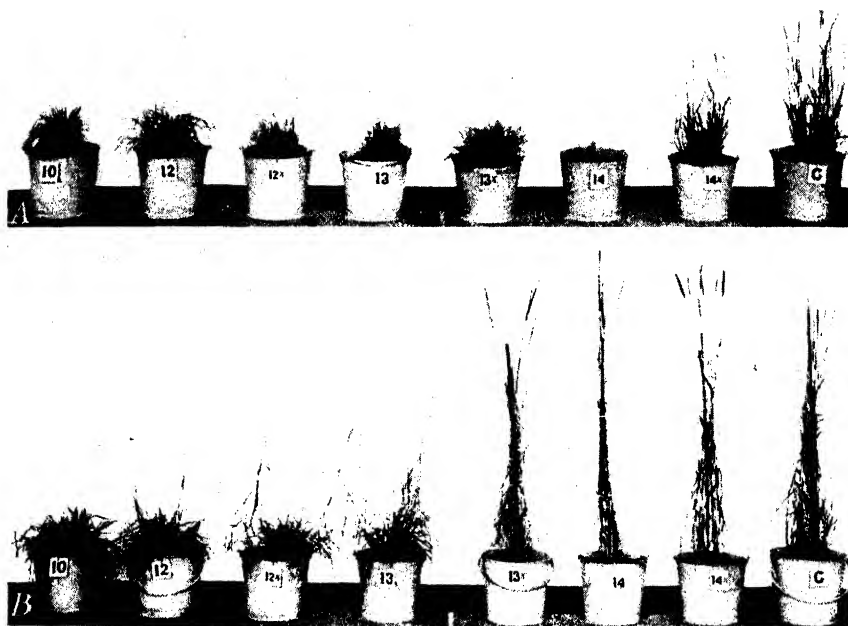


FIGURE 6.—Responses of two American strains of timothy to various constant daily photoperiods (hours) indicated, at Washington, D. C. Controls (C) received full length of day. A, Strain F. C. No. 3937 (Huron): Heading and flowering occurred only in response to lengths of day of 14.5 hours or longer; heads appeared in response to 14.5 hours of light June 28, and pollen appeared July 12. The full length of day (C) produced heads June 18 and pollen June 30. Photographed June 29, 1937. B, Strain F. C. No. 11901 (Marietta): There was weak decumbent stem growth even in response to 12 hours of light, flowering becoming normal with 13.5 hours of light and for all longer periods. Plants receiving 12 hours of light daily produced heads July 1; with 12.5 hours, heads appeared June 14 and pollen July 8; with 13 hours, heads appeared June 21 and pollen July 25; with 14 hours, heads appeared June 1 and pollen June 12; with 14.5 hours, heads appeared May 22 and pollen June 9. The full length of day (C) produced heads May 28 and pollen June 8. This strain has a much lower critical length of day for flowering than No. 3937, and for that reason is an earlier strain better adapted to more southerly sections of the timothy belt. Photographed June 28, 1937.

strains are of the early type. Of the two, 3937 (fig. 6, A) has a much higher critical length of day for flowering than 11901 (fig. 6, B), since the former was unable to flower in response to 13.5 hours of daylight each day, while the latter showed some indications of heading in response to daily light periods of 12 hours. However, the heads of F. C.

11901 showed excessive proliferation until daylight periods of 13.5 hours were experienced. The flower stems of both varieties became strictly erect in response to the full length of day of the Washington region, as well as the daily light periods of 16 and 18 hours that were obtained by the use of supplemental artificial light from sunset on.

TABLE 9.—*Responses of two American strains of timothy to different constant daily light periods*

F. C. NO. 3937 (HURON)

Daily light period	Date when—				Tallest stem	Flower stems	Growth behavior
	First heads appeared	Pollen appeared	First glumes were straw-colored	Half of heads were straw-colored			
<i>Hours</i>					<i>Inches</i>	<i>Number</i>	
10	None	None	None	None	None	None	Heavy, dark-green leafage only.
12	do	do	do	do	None	None	Do.
12.5	do	do	do	do	None	None	Do.
13	do	do	do	do	None	None	Do.
13.5	do	do	do	do	None	None	1 stem; no heading Aug. 13.
14							Plant died July 24, at 7 inches.
14.5	June 28	July 12	Aug. 4	Aug. 16	38		
16	May 28	June 8	June 28	July 1	37		Stems erect.
18	May 23	do	do	July 10	38		Do.
( <sup>1</sup> )	June 18	June 30	Aug. 4	Aug. 13	45		Stems strictly erect.

F. C. NO. 11901 (MARIETTA)

10	None	None	None	None		None	3 short stems, mostly leafage.
12	July 1	do	do	do	28	6	Inflorescence with excessive proliferation; no normal florets.
12.5	June 14	July 8	July 26		32		Inflorescence with much proliferation, remaining green Aug. 11.
13	June 21	None	July 25		36		Inflorescence with much proliferation.
13.5	June 5	June 16	July 19	July 30	50		No proliferation of inflorescence.
14	June 1	June 12	July 12	July 29	54	( <sup>2</sup> )	Do.
14.5	May 22	June 9	July 7	July 24	47	( <sup>2</sup> )	Do.
16	May 23	June 5	June 28	July 6	55	( <sup>2</sup> )	No proliferation of inflorescence; stems erect.
18	May 20	do	June 22	June 30	56	( <sup>2</sup> )	Do.
( <sup>1</sup> )	May 28	June 8	July 2	July 21	55	( <sup>2</sup> )	Do.

<sup>1</sup> Full length of day; plants in buckets.

<sup>2</sup> Many.

It is of interest to note that in response to the full length of day 3937 produced visible heads 21 days and pollen 22 days later than 11901. However, with increase of the daily light periods to 16 and 18 hours, there was no significant difference in the appearance of heads and pollen in the two strains.

While it is evident that both are well adapted to certain sections of the more northerly timothy-growing regions of the country, the day-length behavior as presented in table 9 would indicate that 11901 is an earlier variety, having a somewhat lower critical photoperiod for flowering, and for that reason would be better adapted as a hay grass to more southerly sections of the United States.

Evans has described both the Huron variety (4) and the Marietta variety (5).

#### EUROPEAN STRAINS

The day-length responses of the three European timothy strains discussed in the present paper—Harpenden (England), Russian

(Moscow), and Welsh strain S. 50—are of especial interest since, according to Prof. O. McConkey,<sup>3</sup> of Guelph, Canada, these are all diploid types.

These strains are rather distinctive in growth form, in that they are multitillering types, low-growing with decumbent stems, having the haplocorm (3) poorly developed or absent, and appear to be much better suited to severe grazing conditions, in latitudes where this type of timothy is well adapted, than are the upright-growing, less freely tillering hay grasses.

These three strains of timothy show very typically the normal responses of long-day plants, with a high critical photoperiod for the lower limits of flowering. As a matter of fact the Harpenden strain (table 10), originating in England, is distinctive in not being able to flower or in flowering with a sparse and abnormal growth in response to the longest days of the Washington, D. C., region, where the days are 14.9 hours long from sunrise to sunset at the time of the summer solstice.

Considering the Harpenden strain, it will be noted that the growth, even of leafage, was poor in response to 10 hours of daylight, there being no stem elongation. The growth of leafage improved as the daily light periods increased in length, but stems did not appear until the daily photoperiod had increased to 14.5 hours. Although there was a weak development of stems, these were so declined as to lie flat and stiffly upon the soil. This, however, is a characteristic behavior of these types, even in latitudes as far north as they can be grown.

TABLE 10.—*Responses of three European strains of timothy to different constant daily light periods*

Daily light period	Date when—				Tallest stem	Flower stems	Growth behavior <sup>1</sup>
	First heads appeared	Pollen appeared	First glumes were straw-colored	Half of heads were straw-colored			
Hours					Inches	Number	
10	None	None	None	None	None	None	Growth poor; leafage only; no stems elongated.
12	do	do	do	do	None	None	Growth of leafage only; no stems elongated; better than at 10-hour period.
12.5	do	do	do	do	None	None	Growth of leafage only; better than at 12-hour period; no stem elongation.
13	do	do	do	do	None	None	Growth of leafage only; better than at 12.5-hour period; no stem elongation.
13.5	do	do	do	do	None	None	Good growth of leafage only; no stem elongation.
14	do	do	do	do	None	None	Do.
14.5	do	do	do	do	None	None	A few stems elongated, but so declined as to lie flat upon the soil.
16	May 26	June 11	June 25		13	62	Poor stem growth, these very short, decumbent at base; heads small.
18	May 23	June 4	June 22	July 7	11	57	Better stem elongation, but decumbent at base.
(2)	July 11	July 20	Aug. 5	Aug. 16	10	27	1 or 2 stems only headed, but almost flat upon the ground.
(3)	None	None	None	None	None	None	Mostly leafage; 3 stems only, elongated and had not flowered July 15; stems very decumbent.

<sup>1</sup> See footnotes at end of table.

<sup>2</sup> Oral statement.

TABLE 10.—*Responses of three European strains of timothy to different constant daily light periods—Continued*

## RUSSIAN (MOSCOW) STRAIN

Daily light period	Date when—				Tallest stem	Flower stems	Growth behavior
	First heads appeared	Pollen appeared	First glumes were straw-colored	Half of heads were straw-colored			
<i>Hours</i>					<i>Inches</i>	<i>Number</i>	
10	None	None	None	None	None	None	Leafage only; no stem elongation.
12	do	do	do	do	None	None	Do.
12.5	do	do	do	do	None	None	Do.
13	do	do	do	do	None	None	Do.
13.5	do	do	do	do	None	None	Do.
14	do	do	do	do	None	None	Good growth leafage; no stem elongation; stems very decumbent.
14.5	June 18	June 29	Aug. 20		21	14	Very decumbent stems.
16	May 19	June 3	June 18	June 30	22.5	34	Good growth, but stems very decumbent.
18	May 16	June 1	do	do	27.5	35	Do.
( <sup>2</sup> )	June 18	June 29	July 24	July 3	22.5	53	Do.
( <sup>3</sup> )	do	do	July 20	Aug. 5	13	6	Stems decumbent at base.

## WELSH STRAIN No. S. 50

10	None	None	None	None	None	None	Dense leafage only; no stem elongation.
12	do	do	do	do	None	None	Do.
12.5	do	do	do	do	None	None	Do.
13	do	do	do	do	None	None	Do.
13.5	do	do	do	do	None	None	Do.
14	do	do	do	do	None	None	Do.
14.5	July 6	July 30	July 30	Aug. 26 <sup>4</sup>	12	1	Stems (2) prostrate upon ground.
16	May 16	June 1	June 18		18	48	Flower stems very decumbent, prostrate.
18	do	May 28	do	June 30	19	59	Do.
( <sup>2</sup> )	June 18	June 29	July 30	Aug. 9	17.5	33.5	Flower stems more decumbent than for 16- and 18-hour day.
( <sup>3</sup> )	June 27	July 7	July 27	Aug. 12	16	17	Stems very decumbent at base for first 2 or 3 nodes.

<sup>1</sup> Where measurements, etc., do not agree with data of preceding columns, this is because these observations were made at later date, after changes had taken place.

<sup>2</sup> Full length of day; plants in buckets.

<sup>3</sup> Full length of day; plants in the field.

<sup>4</sup> Green.

Although flowering occurred in response to 16 and 18 hours of light, the stems were noticeably short, decumbent at base, and the heads small. However, the 18-hour light period stimulated a better and more normal growth of stems in length and erect habit than the 16-hour light period (fig. 7, A).

It is obvious that the Harpenden strain of timothy is adapted only to far northern latitudes of the United States and Canada, since in such latitudes the number and length of stems, and consequently hay production, would reach their maximum values.

Data in table 10 show that the Russian (Moscow) strain and the Welsh strain S. 50, from Canada, are very similar to the Harpenden strain in their length-of-day responses (fig. 7, A).

Both the Russian (Moscow) strain and the Welsh strain S.50 showed a somewhat better stem elongation than the Harpenden strain, but the stems of both for the most part were decumbent or prostrate although less so when the longer light periods of 16 and 18 hours were afforded the plants.

It is of interest to note that the longer light periods of 16 hours and of 18 hours have caused all three varieties to flower very noticeably

in advance of the controls experiencing the full length of day at Washington, D. C. This hastening of heading has amounted to as much as a month or more.

Since it would appear from the data presented in table 10 that the longest days of midsummer in the Washington region (lat. 39° N.) are



FIGURE 7.—Responses of two European timothy strains to various constant daily photoperiods (hours) indicated. Controls (C) received full length of day. A, Harpenden (England) strain. There was no stem growth or heading until the 14.5-hour photoperiod was experienced; then no heads were produced. Flowering took place as follows: With full length of day (C), plants flowered July 20 (very few heads); 16 hours, June 11; 18 hours, June 4. The stems of all were decumbent. This is a long-day timothy, requiring longer days than are experienced naturally in the Washington, D. C., region (lat. 39° N.). It is adapted to far northern latitudes. B, Welsh strain S.50, from Canada. There was no stem growth on the 10- to 14-hour photoperiods, inclusive. Nearly prostrate stems appeared on the 14.5-hour plants, but none headed. Dates of flowering with other photoperiods were as follows: With full length of day (C), plants flowered June 29; with 16 and 18 hours, May 16. The stems of all were very decumbent, those of the 18-hour plants being least so. This is a long-day strain, adapted only to the long days of high latitudes. Both photographed May 29, 1936.

scarcely above the critical lower photoperiod for flowering in these timothy strains, it is to be expected that flowering will be retarded as compared with that under greater lengths of day such as 16 hours or 18 hours. So long as the plants experience lengths of day below the critical length, purely vegetative expression must be dominant, and

sexual reproduction manifested by heading and flowering can only weakly assert itself or may remain entirely in abeyance.

These three timothy strains represent some of the latest yet tested in this country, and it is plain to see that this characteristic of lateness is dependent upon inherent behavior associated in part with length of day. With respect to stem elongation and flower formation, these strains must await the arrival of particular lengths of day before the reproductive mechanism is initiated or set in motion.

These late strains appear to have originated in England or on the continent of Europe and naturally have been selected to respond to lengths of day occurring in latitudes ranging from 50° to 60°, since southern England is just above the 50° parallel and Moscow, U. S. S. R., is just above the 55° parallel.

The northern boundary of the United States is wholly below latitude 50° N., and much of the best of the timothy-growing belt lies near 40° to 45°. On these parallels, the midsummer days from sunrise to sunset reach their maximum values of 15 hours and 15.3 hours respectively at the summer solstice, June 21. However, the plants experience these maximum lengths of day but briefly, owing to the ceaseless seasonal swing of the lengths of day between minimum values at the winter solstice and the maximum values of the summer solstice.

Data in table 11, showing the duration of the longest days at various latitudes, may be considered in connection with the behavior of the Harpenden strain of timothy, which in the Washington, D. C., region appears scarcely able to produce flowering stems or shows greatly delayed flowering.

TABLE 11.—Hours from sunrise to sunset (intervals of 2 weeks) for latitudes<sup>1</sup> 25° to 60°, during the longer days of the summertime

[Summer solstice and maximum length of day, June 21]

Date	25°	30°	35°	30°	40°	45°	50°	55°	60°
April 21	12.53	13.04	13.17	13.28	13.31	13.48	14.09	14.34	15.08
May 1	13.07	13.21	13.37	13.52	13.55	14.17	14.42	15.15	16.00
May 11	13.18	13.35	13.54	14.11	14.16	14.41	15.14	15.55	16.50
May 21	13.28	13.47	14.09	14.29	14.35	15.04	15.41	16.29	17.83
June 1	13.36	13.58	14.22	14.44	14.50	15.24	16.04	16.58	18.20
June 11	13.40	14.02	14.30	14.52	14.58	15.33	16.19	17.18	18.44
June 21	13.42	14.04	14.32	14.56	15.02	15.38	16.24	17.24	18.54
July 1	13.40	14.02	14.30	14.53	14.58	15.34	16.18	17.18	18.36
July 11	13.36	13.58	14.22	14.45	14.50	15.24	16.04	16.58	18.19
July 21	13.29	13.48	14.12	14.31	14.36	15.06	15.44	16.33	17.41
Aug. 1	13.19	13.35	13.54	14.12	14.16	14.42	15.15	15.55	16.51
Aug. 11	13.07	13.20	13.38	13.51	13.55	14.19	14.43	15.18	16.01
Aug. 21	12.54	13.06	13.18	13.28	13.32	13.49	14.09	14.35	15.09

<sup>1</sup> Approximate geographical positions of various latitudes:

25° N.—Close to tip of southern Florida and central Mexico.

30° N.—Near the southern boundary of southern Louisiana and northern Florida.

35° N.—Near the southern boundary of North Carolina and Tennessee.

39° N.—Latitude of Washington, D. C.

40° N.—Near the southern boundary of Pennsylvania and central Ohio, Indiana, and Illinois.

45° N.—Near the center of Maine, northern Vermont, and Michigan, and southern Minnesota.

50° N.—Near northern France, southern England, central Quebec, and the northern boundary of the United States west of the Great Lakes.

55° N.—Near Moscow, U. S. S. R., southern Scotland and central Canada.

60° N.—Near Leningrad, U. S. S. R., southern Scandinavia, and southern Alaska.

It may, for example, be assumed that this strain is initiated into the flowering condition with a length of day near 15 hours. It is probable that a certain twilight intensity, at least in the evening, may also be operative, thus extending the effective length of day somewhat, for it is well established that quite low intensities when used to extend a

photoperiod of natural daylight beyond the critical length may be effective, as shown by Borthwick and Parker (2).

These authors have shown that, in the case of the Biloxi soybean, with a short day of 8 hours, which is below the critical photoperiod or the formation of flower primordia, an intensity of at least 100 footcandles of illumination must be supplied before flower primordia can arise. If, on the other hand, 8 hours of natural daylight are used and 8 additional hours of Mazda light are then added, making a total of 16 hours of continuous illumination, which is above the critical photoperiod for the initiation of flower primordia, the added light following the 8-hour period of natural light can be as weak as 0.6 footcandle, which appears to be near the lower limit of effective intensity under these conditions.

Kimball's (13) data would indicate that with practically cloudless skies the intensities of civil twilight,<sup>4</sup> beginning at dawn or ending in the evening when the true position of the sun's center is 6° below the horizon, range from about 0.4 footcandle to 33 footcandles. However, the case is somewhat different with the evening twilight, which at that time decreased from about 33 footcandles at sunset to 0.4 at the end of civil twilight. It is evident that practically all this period, amounting to about 35 to 36 minutes morning and evening through the summer season, is effective light in extending the length of day for the Biloxi soybeans.

With respect to the timothy strains under discussion, nothing is known of the actual lower limits of intensity required for flower-bud initiation. However, it may be assumed that both morning and evening twilight may be in part effective, and it is possible that the effective length of day from sunrise to sunset may be extended nearly an hour at Washington, D. C., lat. 39° N., by these two twilight periods.

For convenience 15 hours of illumination may be regarded as the lower limit for final flower development in the Harpenden strain, since heading and flowering were scarcely attained at Washington, D. C. Ignoring civil twilight, which is about 1 hour at Washington, and the effects of which cannot be evaluated for the long-day grasses at the present time, the plants do not receive 15 hours of light until about June 11; and even at the summer solstice the maximum period may not be much in excess of 15 hours of effective light. It will be noted from the data in table 13 that additional Mazda light after sunset, amounting to 200-300 footcandles and bringing the constant daily light periods to 16 and 18 hours, has caused heads to emerge May 26 and May 23, respectively, while the full length of day caused heads to appear July 11, about 7 weeks later. Since the tests began with these photoperiods April 21, about 1 month was required for visible heading, but actual initiation of the flower primordia must have taken place long before, perhaps within a few days from the beginning of the tests. If the rate of growth had been the same in response to the full length of day as in the 16-hour and 18-hour tests, the daily photoperiods of 15 hours, beginning about June 11, should have caused heading about July 11, as was observed.

Within the timothy belt more properly speaking, lying between

<sup>4</sup> The term "civil twilight" signifies that period after sunset and before sunrise during which there is enough light on clear days for ordinary occupations. Civil twilight ends at night and begins at dawn when the sun is about 6° below the horizon, and averages close to 35 to 36 minutes in length at Washington, D. C., for the year.



latitudes  $45^{\circ}$  and  $50^{\circ}$ , there is a noticeable increase in length of day, as shown in table 11. With respect to the original assumption of 15 hours as the shortest photoperiod capable of initiating sexual reproduction in the Harpenden strain, it may be noted that 15 hours of light (with twilight of about 1 hour or more additional) begins near May 11 for latitude  $45^{\circ}$  and that the daily light period remains above 15 hours until near August 1, i. e., it begins about 1 month earlier than for latitude  $39^{\circ}$  and persists about 1 month later. For latitude  $50^{\circ}$ , the comparable 15-hour photoperiod is advanced to near May 1; for latitude  $55^{\circ}$ , it is advanced to near April 21; and for  $60^{\circ}$ , nearer April 11, persisting until correspondingly later dates.

In a consideration of the ecological factor of length of day and its effects upon plant growth and reproduction in northern latitudes, it is important to consider these seasonal relationships brought about by changes from lower to higher latitudes. Not only may the critical length of day from sunrise to sunset be advanced to a much earlier date, thus giving a much longer period with lengths of day above the critical length before and after the summer solstice has arrived, but in very high latitudes the evening twilight lengthens appreciably, adding considerably to the effective length of day for some plants.

Thus it is plain why a timothy strain like Harpenden may become delayed in heading and flowering in the latitude of Washington, D. C. (lat.  $39^{\circ}$  N.), where the day length is perhaps unfavorable to the reproductive activities. At higher latitudes to which it has been adapted by selection and where lengths of day are perhaps much above the critical length for relatively long periods before the summer solstice, such a strain may become commercially valuable. Typical long-day plants, then, such as Harpenden, Russian (Moscow), and Welsh S. 50 strains of timothy, other conditions being favorable, are hastened rather than delayed in their flowering responses when carried to higher latitudes. This behavior is well shown when these strains have been subjected to constant daily light periods of 16 and 18 hours, affording photoperiods comparable to natural daylight durations experienced in latitudes  $55^{\circ}$  to  $60^{\circ}$ .

It is to be understood that the very decumbent growth form of these particular timothy strains is not related to a particular length of day, since this behavior is known to manifest itself in these forms as far north as they can be grown in Scotland and on the European mainland. While this expression appears to be a genetic character in these particular diploid strains, there is reason to believe that it is more or less modifiable, depending upon external environmental influences. There seems to be no logical reason why upright habit could not be developed by intelligent selection in some of these diploid strains.

It would appear that the biotic factor of intensive grazing has given these forms an advantage in pastures which they otherwise would not have, in competition with the more upright hay types.

#### BULBOUS BLUEGRASS

Bulbous bluegrass (*Poa bulbosa*) is unique in that some or all of the florets become converted into bulbils, a form of vegetative expression.

Tests to determine the response of *Poa bulbosa* to various lengths of day were made with small clumps from overwintering sod in the field and also with plantings of the small bulbils of the inflorescence.

The responses of the two groups were not the same. In the case of the overwintering sod material, there was no indication of flowering in any test and the plants appeared entirely brown as if mature and dead, from the shortest to the longest days, about the first of June.

The behavior of the young plants grown from the planted bulbils is shown in table 12. It will be seen from these data that stem and head formation, attended by the appearance of bulbils in the florets, did not occur until light periods of 13.5 hours were experienced. These bulbils continued to form for the most part in all photoperiods longer than 13 hours.

TABLE 12.—*Responses of bulbous bluegrass to different daily light periods*

Daily light period	Date when—		Tallest stem	Growth behavior
	First heads appeared	Pollen appeared		
<i>Hours</i>			<i>Inches</i>	
10	None	None	None	Leafage apparently dead, brown July 12.
12	do	do	None	Leafage apparently dead, brown July 29.
12.5	do	do	None	Leafage apparently dead, brown June 24.
13	do	do	None	Leafage apparently dead, brown July 3.
13.5	June 3		11	Small bulbils appeared in the heads and matured June 21. Plants apparently dead, brown, June 25.
14	None	None	7	Stems developed but no heads or bulbils; apparently dead, brown, June 25.
14.5	June 1		11	Small bulbils formed in the heads June 7, maturing June 30; apparently dead, brown July 9.
16	do		16	Small bulbils formed in the inflorescence; maturing June 15; plants apparently dead, brown Aug. 19.
18	May 28		16	Bulbils formed in inflorescence June 7; maturing June 21. Bulbils in this test much larger than in the test where 16 hours of light was used.
(1)	June 2		13	Small bulbils forming June 11; fully mature, brown June 21.

<sup>1</sup> Full length of day; plants in buckets.

The most striking difference observed in any test was associated with the light period of 18 hours, for the individual bulbils were strikingly large and even exceeded in size those formed with 16 hours of light. The tallest stems were formed in response to the 16-hour and the 18-hour photoperiods. By midsummer practically all the plants had become brown.

The behavior of the *Poa bulbosa* material was not as clearly expressed as has been the case with most grasses, and leaves much yet to be understood. Under the conditions of the tests reported there seemed to be no particular length of day when all the florets were sexually perfect, with functional stamens and pistils, since bulbils were always present.

Different strains of *Poa bulbosa* appear to behave differently with regard to the production of normal inflorescences, and these differences may have a genetic basis rather than representing simple response to some cultural condition.

#### CREEPING BENT

Two forms of a species of creeping bent (*Agrostis palustris*) have been studied, one known as Washington bent, the other as Metropolitan bent. These bents are used for lawns and putting greens. Both were studied from small clumps of overwintering field material.

The Metropolitan strain has shown a behavior rather different from that of the Washington strain. The former never produced heads in any test with photoperiods ranging from 10 hours to 18 hours; remaining green throughout the summer.

However, while there was no heading, growth was vigorous and stolons appeared in all the longer photoperiods, beginning with the 13-hour tests. It would appear that the longer light periods favor runner development, which is a prostrate type of vegetative stem, rather than the shorter photoperiods below 13 hours.

The behavior of the Washington bent (table 13) has been somewhat different, since this form was induced to flower on May 23 and 27, in response to 16 and 18 hours of light respectively. As a matter of fact, the plants flowered in no other test.

TABLE 13.-- Responses of Washington creeping bent to different constant daily light periods

Daily light period	Date when--		Tallest stem	Growth behavior
	First heads appeared	Pollen appeared		
<i>Hours</i>			<i>Inches</i>	
10	None	None	None	A mat of short very compact green leafage only, Aug. 4.
12	do	do	None	A dark-green velvety growth of leafage only, Aug. 10.
12.5	do	do	None	A dark-green velvety growth of leafage only, Aug. 17.
13	do	do	None	A dark-green growth of leafage; short stolons appearing Aug. 11.
13.5	do	do	None	A dark-green growth of leafage; short stolons as in plants of 13-hour test Aug. 13.
14	do	do	None	Similar in growth to plants of 13.5-hour test.
14.5	do	do	None	Do.
16	May 23	June 9	16	Tips of heads browning July 15; half of heads brown July 22; plants green Aug. 19.
18	May 27	June 7	16	Tips of heads browning June 24; half of heads brown June 30; plants green Aug. 19.
(1)	None	None	None	Green leafage only, Aug. 18.

<sup>1</sup> Full length of day; plants in buckets.

The vegetative expression of the plants varied also, depending upon the photoperiod. While a dark-green velvety leafage was formed in response to all the tests where flowering did not occur, stolons became evident in response to 13 hours of light, as in the case of the Metropolitan strain, and were formed under all the longer photoperiods where a purely vegetative growth obtained.

#### INDIAN GRASS

One of the most interesting and beautiful grasses of the Washington region is the wild native Indian grass (*Sorghastrum nutans*). Aside from any consideration of usefulness in man's economies, this stately grass always commands attention in August, with its big, drooping, yellowish-brown, mobile panicles brightened with the rich yellow gold of the anthers.

Not only is it a conspicuous feature of our eastern wasteland successions, but it is a common constituent of the tall-grass prairies of the eastern Great Plains region (fig. 8).

In its length-of-day responses it has shown an almost classic behavior with the sharpness of its various responses to particular photoperiods.

Data in table 14 show that the plants remained strictly vegetative, with no stem development, until 13.5 hours of constant daily light had been experienced. On shorter photoperiods the buckets were filled with a dense leafage only, which, in the case of the 10-hour photoperiod, was very stiffly erect and had attained a height of only 7 inches in October as compared with 15 inches for the photoperiods of 12, 12.5, and 13 hours.



FIGURE 8.—Responses of Indian grass (*Sorghastrum nutans*) to various constant daily photoperiods (hours) indicated. Control (C) received full length of day. With 10- to 13-hour photoperiods plants did not flower but produced masses of stiff erect foliage. The critical photoperiod for flowering is near 13.5 hours; flowering occurred August 29. Plants receiving 14 and 14.5 hours and full length of day flowered August 20, August 29, and August 15, respectively. This grass appears to have a rather sharp lower critical photoperiod for flowering. Photographed August 31, 1938.

TABLE 14.—Responses of Indian grass to different constant daily light periods

Daily light period	Date when—		Tallest stem	Flower stems	Growth behavior
	First heads appeared	Pollen appeared			
Hours			Inches	Number	
10	None...	None	None	None	Dense, stiffly erect leafage only, 7 inches high Oct. 2.
12	...do...	...do	None	None	Dense leafage filling pail; 15 inches high Oct. 2.
12.5	...do...	...do	None	None	Do.
13	...do...	...do	None	None	Do.
13.5	Aug. 11	Aug. 29	46	10	Growth vigorous, normal.
14	...do...	Aug. 20	42	17	Do.
14.5	...do...	Aug. 29	64	21	Do.
16	Oct. 17	Oct. 20	56	9	Heads long remained in the boot, not heading till mid-October; of 13 stems only 9 produced heads.
18	Oct. 27	Oct. 31	50	6 (?)	Heads long remained in the boot, not heading till late October; of 22 stems only 6 produced heads.
(1)	Aug. 1	Aug. 15	56	19	Growth vigorous, normal.

<sup>1</sup> Full length of day; plants in buckets.

Heading and flowering occurred at approximately the same date in response to 13.5, 14, and 14.5 hours and to the full length of day.

In response to 16 and 18 hours of light, however, heading and flowering were delayed until late October, although the final heights

attained were about equal to those of the controls receiving the full length of day and to all other tests in which the plants flowered.

In the case of the 16- and 18-hour photoperiods, the heads long remained in the boot, indicating that these photoperiods and conditions were not entirely favorable to normal flowering. In the case of the 16-hour plants, a total of 13 stems developed, but only 9 headed and flowered; the 4 remaining did not appear to have heads in the boot. The plants experiencing the 18-hour photoperiod produced 16 sterile stems and only 6 flowering stems. It is obvious that these long photoperiods have not only greatly delayed anthesis, but have resulted in a large proportion of sterile stems or at least such as are incapable of maturing an inflorescence.

These plants were brought into the greenhouse October 12. At this season of the year the natural length of day is only 11.22 hours, and it is evident from the data of table 14 that this photoperiod is much too short for stem formation in this grass. Cessation of stem growth at this stage is probably incident to these unfavorable short photoperiods, since at this season the day length from sunrise to sunset is far below the critical photoperiod for stem formation and flowering, which is 13.5 hours in the various tests.

The natural range of this grass may now be considered in the light of the responses shown in the data of table 14.

The normal range of this species is from Maine, Quebec, and Manitoba, on the north, to Florida, Texas, Arizona, New Mexico, and Mexico, on the south. Roughly this grass finds its range near the northern and southern limits of the United States, embraced between latitudes 25° to 30° on the south and 45° to 50° on the north.

Since the tests have shown that a 13.5-hour photoperiod is favorable to flowering, it may be seen by reference to table 11 that 25° is very near the lower latitudinal limit of distribution of this grass, since the maximum length of the day is barely more than 13.4 hours.

Latitudes 45° and 50° have a mean day length considerably under 15.5 hours and above 16 hours, respectively, from May 21 to July 11. It is probable that latitude 50° is nearer the northern limit of distribution of *Sorghastrum nutans*, since the tests have shown that a photoperiod of 16 hours causes great delay in time of flowering.

These assumptions would only apply to the particular strain used in this test. It is always possible that the more northern material and the more southern material may include strains that are better adapted to the longer and shorter days, respectively, than is the particular type tested from middle latitudes.

In the more northern latitudes the factor of lower mean temperatures during the growing season will tend to work in the direction of furthering delay brought about by unfavorable lengths of day. In the case of such long-day plants as Indian grass, both factors combine to delay flowering and thus to define the northern limits of range. The southern limits will depend more purely upon the factor of the length of day alone, since the temperature means are increasing.

Since Indian grass is shown to be unable to flower or to flower with great delay when the days are either too short or too long, it may be considered to belong to that group of plants termed "intermediate" which the senior author has reported upon in a recent paper (1).

## BOTTLEBRUSH

The genus *Hystrix*, to which bottlebrush belongs, is striking in having a very discontinuous distribution, one small group of two species being native to North America, another species native to Australia, and still another species native to Asia.

The eastern American species (*Hystrix patula*) is a woodland grass, characteristic of rocky slopes of the higher terrain. It flowers in June and contributes an element of beauty to its shaded woodland haunts with its bluish glaucous stems and long-awned, spreading florets loosely arranged in graceful spikes, but it has no known commercial value as a forage grass.

In its length-of-day response, it would appear to be a grass of the long-day class, since lengthened photoperiods favor its sexual reproduction, and the lower critical photoperiod for flowering is about 12 hours (table 15).

With only 10 hours of light daily, it produced mostly leafage and a single nearly stemless head among the leaves. With 12-hour daily photoperiods many stems were produced but these were short and some even failed to head.

TABLE 15.—Responses of bottlebrush to different constant daily light periods

Daily light period	Date when —		Tallest stem	Flower stems	Growth behavior
	First heads appeared	Pollen appeared			
Hours			Inches	Number	
10	June 20	None	None	None	One head practically stemless; mostly leafage.
12	June 16	June 30	15	( <sup>1</sup> )	Heads very low; many stems, some not heading.
12.5	June 7	June 21	17	( <sup>1</sup> )	All mature but dead Sept. 27.
13	June 15	July 1	25	( <sup>1</sup> )	Do.
13.5	June 13	June 30	30	( <sup>1</sup> )	Do.
14	June 21	July 1	30	( <sup>1</sup> )	Tops dead Sept. 27; small shoots below appear.
14.5	June 16	June 30	29	( <sup>1</sup> )	Green heads; small lateral green shoots appear.
16	June 13	June 25	26	( <sup>1</sup> )	Vigorous, normal growth.
18	June 9	do	31	( <sup>1</sup> )	Do.
( <sup>2</sup> )	June 20	July 1	33	( <sup>1</sup> )	Do.

<sup>1</sup> Many.

<sup>2</sup> Full length of day; plants in buckets.

The length-of-day response of this grass, more especially its behavior in response to 16- and 18-hour photoperiods, would indicate a natural distribution well northward, and as a matter of fact its northern range extends at least to the northern boundary of the United States, near lat. 50° N. (fig. 9.)

## EASTERN GAMAGRASS

Eastern gamagrass (*Tripsacum dactyloides*), whose length-of-day responses are shown in table 16, on the basis of its reactions to the various photoperiods, would appear to be indeterminate in its classification.

There has been no marked change in the time of flowering from the shortest photoperiods of 10 hours to the longest photoperiods of 18 hours. There appears to be some delay in heading and anthesis with the longer photoperiods, indicating that the shorter are a little more favorable to early sexual reproduction.

TABLE 16.—Responses of eastern gamagrass to different constant daily light periods

Daily light period	Date when—			Tallest stem	Growth behavior
	First heads appeared	Pollen appeared	Glumes brown		
<i>Hours</i>				<i>Inches</i>	
10	June 2	June 11	June 23	64	Both staminate and pistillate spikelets; fruitful.
12	June 4	June 14	June 21	56	Do.
12.5	June 7	do	do	45	Do.
13	do	do	June 30	67	Do.
13.5	do	June 15	June 23	55	Do.
14	June 20	June 28	July 10	56	Do.
14.5	June 21	do	July 7	60	Do.
16	July 13	July 18	July 24	63	Do.
18	June 23	June 30	July 6	54	Do.
(1)	June 21	June 28	July 3	65	Do.

1 Full length of day; plants in buckets.

With respect to growth of stems in height and number and in the proportions of the unisexual staminate or pistillate florets, the responses of this species to the various photoperiods, whether long or short, were remarkably uniform.

On the basis of its readiness to grow and to flower normally with only 10 hours of light daily (fig. 10), it is indicated that this species is adapted to the length of day of tropical regions. As a matter of fact this grass in its southward range occurs in the West Indies, Mexico, and Brazil, the northern portion of which lies between lat. 5° N. and 0° (the Equator).



FIGURE 9.—Responses of bottlebrush (*Hystrix patula*) to various constant daily photoperiods (hours) indicated. Control (C) received full length of day. This grass shows little variation in response to different constant light periods, except at the lowermost limits of the shorter photoperiods. Plants receiving 10 hours of light daily finally produced a very weak panicle June 20; but florets produced no pollen. Dates of flowering with various photoperiods were as follows: With 12 hours, plants flowered June 30; 12.5 hours, June 21; 13 hours, June 15; 13.5 hours, June 13; 14 hours, June 21; 14.5 hours, June 16; full length of day (C), June 11. This grass, a native of the Washington, D. C., region, behaves somewhat as a long-day type with a very low critical photoperiod unfavorable to flowering. Photographed August 31, 1938.

The responses of this grass to the photoperiods of 16 and 18 hours would indicate that it should have a far northern distribution on the basis of the length-of-day factor alone, but in nature its northernmost limits appear to be Massachusetts, New York, Michigan, Illinois,

Iowa, and Nebraska. It is probable that other factors, such as low mean temperatures or great extremes of cold, may be a check to its northern distribution.

As the data stand, this species is indeterminate in its day-length responses, but with tendencies to delay flowering somewhat with very long photoperiods, indicating the more typical behavior of a short-day type of plant.

The genus *Tripsacum* is American and is represented by several species, most of which are limited to the extreme southern part of



FIGURE 10.—Responses of eastern gamagrass (*Tripsacum dactyloides*) to various constant daily photoperiods (hours) indicated. Control (C) received the full length of day at Washington, D. C. There was little difference in dates of flowering with various photoperiods: With 10 hours, plants flowered June 11; 12 hours, June 14; 12.5 hours, June 14; 13 hours, June 14; 13.5 hours, June 15; 14 hours, June 28; 14.5 hours, June 28; full length of day (C), June 28. At the time of flowering the heights of the plants were very constant, the mean being about 60 inches. The plants were photographed June 29, the earlier flowering plants of the shorter photoperiods elongating in the meanwhile, giving the difference in height shown in the picture. The tendency of this species to show delayed flowering on the longer photoperiods would indicate the more typical behavior of a short-day plant. Photographed June 29, 1937.

North America or to Central America, where some are cultivated for forage.

Eastern gamagrass is sporadic in its occurrence, preferring low, moist soils, and is not of great importance in American pastures.

#### SUMMARY

The length-of-day responses of *Poa compressa*, *P. pratensis* (3 strains), *P. bulbosa*, *Dactylis glomerata*, *Muhlenbergia mexicana*, *M. schreberi*, *Phalaris arundinacea*, *Bromus inermis*, *Phleum pratense* (5 strains), *Agrostis palustris* (2 strains), *Sorghastrum nutans*, *Hystrix patula*, and *Tripsacum dactyloides* were studied at the Arlington Experiment Farm at Arlington, Va.

The plants experienced the natural daylight for all photoperiods except those of 16 and 18 hours' duration, which required from sunset



the use of Mazda light from four 200-watt bulbs with reflectors, to supplement the normal length of day, giving an intensity at 1 foot, the distance at which the plants were maintained, of 300 to 400 foot-candles. Automatic time switches were used to turn the lights on and off at the proper time, these being adjusted from time to time to compensate for changes in the natural daylight period before and after the summer solstice.

For all constant photoperiods shorter than the full length of day, a portion of the daylight was excluded each day by keeping the plants in ventilated lightproof houses.

In the case of Canada bluegrass (*Poa compressa*), the critical photoperiod for heading and flowering is rather sharply delimited at 13.5 hours, while in the case of the very late timothy strain, Harpenden, flowering is attained with difficulty in response to the longest days (14.9 hours) of the Washington region.

The Kentucky bluegrass (*Poa pratensis*) strains, early, medium-late, and late, show more or less well-defined differences in growth behavior in their length-of-day responses, but under the conditions of the experiments flowering responses have not been so striking. The early strain, heading and flowering 3 to 4 weeks in advance of the two later strains, represents some inherent strain difference. Various degrees of the decumbent habit of stem growth were shown on the shorter day lengths by all the strains. It is evident that Kentucky bluegrass finds its best development, at least with respect to erect habit of growth, in the longer days and cooler temperatures of the more northern latitudes.

The time of flowering and heading of orchard grass (*Dactylis glomerata*) was but little affected by any of the photoperiods used, except that of 10 hours. This photoperiod was characterized by dense tussocks of leafage only.

Wirestem muhly (*Muhlenbergia mexicana*) showed nothing particularly distinctive in its responses to the various constant daily photoperiods with respect to time of heading and flowering. Ten hours of light daily caused very low growth, with flowering stems so short that they appeared almost on the ground. The longer photoperiods resulted mainly in greater elongation of the flower stems and a more erect habit of growth.

Nimblewill (*Muhlenbergia schreberi*) showed no very marked response to increased duration of the photoperiods until artificial light was used to extend the day to 16 hours and 18 hours, respectively. These photoperiods appeared to stimulate a very characteristic prostrate type of growth, and flowering was delayed a month or more.

Reed canary grass (*Phalaris arundinacea*) is a long-day grass, but its flowering extends down to include daily photoperiods only 12.5 hours in length. Flower stems were sparsely produced until 14.5 hours of light daily were experienced, and all were decumbent. With 10 and 12 hours of light daily the plants produced leafage only.

Smooth brome grass (*Bromus inermis*) is likewise a long-day type of plant. There was no flowering in response to photoperiods of 10, 12, 12.5, and 13 hours, dense leafage only being produced. With increase of the daily photoperiod beyond 13 hours, flowering became more profuse and the stems longer and more erect in growth.

The various strains of timothy (*Phleum pratense*) have shown more or less marked differences in response to the different photoperiods. The American strains F. C. 3937 (Huron) and F. C. 11901 (Marietta)

are early flowering types. Of the two, 3937 has a much higher critical length of day for flowering than 11901, since the former was unable to flower in response to 13.5 hours, while the latter showed indications of heading at 12 hours. The flower stems of both became completely erect in response to the natural length of day at Washington, D.C., and to the longer photoperiods of 16 and 18 hours obtained by using artificial light. It is thus indicated that 11901 is an earlier variety, and for that reason is perhaps better adapted to more southern sections of the United States.

Three European strains of timothy were studied, i. e., Harpenden, Russian (Moscow), and Welsh strain S. 50. All show typically the behavior of long-day plants, with a high critical for the lower limits of flowering, and all are very similar in their length-of-day responses. These three timothy strains represent some of the latest yet tested in the United States, and are evidently best suited to high northern latitudes here, as well as in England or on the continent, where they originated.

Bulbous bluegrass (*Poa bulbosa*) was grown from bulbils and also from clumps taken from overwintering sod. The responses of these were somewhat different, since the sod material never flowered in any photoperiod. Young plants from bulbils did not produce flowers or bulbils until a light period of 13.5 hours was experienced, and these formed in all photoperiods longer than 13.5 hours.

An accentuation of stolon formation was found to be associated with longer days in the tests with the two creeping bents (*Agrostis palustris*), Metropolitan and Washington, and in the case of the Washington bent flowering was also associated with the longer days.

Indian grass (*Sorghastrum nutans*) is very sensitive to certain changes in length of day, the lower critical limit for flowering being about 13.5 hours. This species is greatly delayed in heading and flowering when the photoperiods have been increased to 16 or 18 hours. In its inability to flower in response to short days and in its greatly delayed or partially inhibited flowering with photoperiods of 16 hours or longer, this plant appears to be of the intermediate class in its flowering behavior, that is, there is complete or partial inhibition of flowering when the days are too short or too long.

Bottlebrush (*Hyssrix patula*) is a woodland species, flowering most readily in response to long day and therefore finding its best development in middle and northern latitudes, as its natural range would indicate.

Gamagrass (*Tripsacum dactyloides*) is very unresponsive to changes in length of day, except when photoperiods are very long. This species grows particularly well in photoperiods as short as 10 hours, and this behavior is exemplified by its range southward into Mexico and into Brazil where truly equatorial lengths of day prevail.

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# FURTHER STUDIES ON THE OAT SMUTS, WITH SPECIAL REFERENCE TO HYBRIDIZATION, CYTOLOGY, AND SEXUALITY<sup>1</sup>

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## INTRODUCTION

Hybridization in the oat smut fungi has been demonstrated by a number of investigators (2, 3, 7, 8, 10)<sup>2</sup> and in some instances (5, 7, 10) pathogenically distinct races of *Ustilago avenae* (Pers.) Jens. and *U. levis* (Kell. and Sw.) Magn. have been produced artificially by this process. In 1931, the writer (3) described a buff smut of oats which appeared in hybrid material but which later was found to have arisen through mutation in *U. levis* (5). It was further shown that the buff smut fungus crosses readily with *U. avenae* and *U. levis* and that the buff character is inherited as a recessive (4, 6). Consequently, in such hybrids a buff F<sub>2</sub> segregate is homozygous for this character. Therefore, by selecting a buff segregate on a variety immune from the buff parent and susceptible to the *U. avenae* or *U. levis* parent it is possible to obtain a race of the buff smut that possesses the pathogenicity of the other parent or of both parents. Such a hybrid race was reported by the writer (5) in 1936, and on this basis it appeared theoretically possible to produce, by hybridization, a race of the buff smut for every race of *U. avenae* and *U. levis* available. Accordingly, investigations were undertaken to determine the validity of this theory. Studies also have been made on nuclear behavior in the buff smut, on the inheritance of sorus type in two races of *U. avenae*, and on the process of sporidial fusion in all of the oat smuts. The results of these investigations are reported in this paper.

## MATERIAL AND METHODS

The isolation of single sporidia and the determination of compatible combinations of monosporidial lines were accomplished by the methods previously described (4). Inoculations were made either by the method used formerly (3) or by the partial vacuum method described by Allison (1). Hybrid chlamydospores were obtained by inoculating Anthony (C. I.<sup>3</sup> 2143) oats with paired monosporidial lines of *Ustilago avenae* and the buff smut and *U. levis* and the buff smut. Because of the high degree of sterility in sporidia from hybrid chlamydospores (3, 4), the F<sub>1</sub> spores were used to inoculate differential varieties, and buff F<sub>2</sub> segregates were selected from varieties immune from the buff parent. Inoculum of succeeding generations of the F<sub>2</sub> buff selections was taken from the same varieties on which the F<sub>2</sub> segregate appeared.

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<sup>2</sup> Italic numbers in parentheses refer to Literature Cited, p. 240.

<sup>3</sup> C. I. refers to accession number of Division of Cereal Crops and Diseases.

Two races of *Ustilago avenae* that produce distinctly different sorus types were used to study the heritability of this character. Hybrid spores were obtained in the manner described above, and since the sporidia from these  $F_1$  spores will grow in culture, inoculations were made with combinations of monosporidial lines from the  $F_1$  chlamydospores to obtain the  $F_2$  population. Complete sets of four monosporidial lines were obtained from five  $F_1$  chlamydospores; inoculations were made with the compatible combinations within each set, and one set was used in backcrosses to the parent lines.

The nuclei of the buff smut fungus in several stages of development were stained with Heidenhain's iron-alum hematoxylin. The procedure described previously (4) was used, except that the material was destained with acid alcohol instead of iron-alum.

The process of sporidial fusion was studied by mating sporidia of opposite sex in pairs on plain water agar. The pairings were made with the aid of a Chambers' micromanipulator and the sporidia in each pair were distinguished from each other by difference in size, i. e., small sporidia were selected from one line and mated with relatively large sporidia selected from the other line.

## RESULTS

### PHYSIOLOGIC RACES OF BUFF SMUT

Seven races of the buff smut have been identified on the basis of the differential reaction of certain oat varieties. One of these races arose through mutation, one represents a field collection, and five were produced by hybridization. The results of pathogenicity tests on which the separation of the races was based are presented in table 1. The origin and distinguishing characteristics of each race are as follows:

*Race 1.*—Mutant from *Ustilago levis* collected in McLeod County, Minn., in 1930. As shown in table 1, Canadian is highly susceptible to this race and 17 percent smut was produced on Richland.

*Race 2.*—An  $F_2$  segregate on Gothland from a hybrid between buff race 1 and a Gothland race of *Ustilago avenae*. Differs from race 1 primarily by the susceptible reaction of Gothland to race 2.

*Race 3.*—Collected by Harland Stevens from a row of oats in a seed stock nursery grown for T. R. Stanton at Aberdeen, Idaho, in 1935, the seed of which was obtained from a Chicago, Ill., grain elevator. This race differs from race 1 by the susceptible reaction of Gothland and from race 2 by the susceptibility of Richland.

*Race 4.*—An  $F_2$  segregate on Black Mesdag from a hybrid between buff race 1 and a Black Mesdag-Monarch race of *Ustilago levis*. This race is characterized by its capacity to infect Black Mesdag.

*Race 5.*—An  $F_2$  segregate on Monarch from a hybrid between buff race 2 and a Black Mesdag-Monarch race of *Ustilago levis*. Its virulence on Gothland, Monarch, and Black Mesdag distinguishes this race from the others. It is notable that Monarch is more susceptible and Black Mesdag is less susceptible to this race than to the *U. levis* parent and that Gothland is less susceptible than it is to the buff parent (table 1).

*Race 6.*—An  $F_2$  segregate on Trojan from a hybrid between buff race 1 and a Monarch-Richland race of *Ustilago levis*. This race is outstanding for its capacity to infect Alabama Red Rustproof, which is resistant to both parent races. It is definitely less virulent on Monarch than the *U. levis* parent and about equal in virulence to both parent races on Richland.

*Race 7.*—An  $F_2$  segregate on Fulghum from a hybrid between buff race 1 and a Monarch-Fulghum race of *Ustilago levis*. This race is characterized by its virulence on Fulghum and Monarch and, to a slight degree, on Black Mesdag.

TABLE 1.—Differential host reaction to parent and hybrid races of buff smut of oats

Parent and hybrid races of oat smut	Race No.	Percentage of smut in host testers—						Alabama selection Red Rust-proof (C.I. 1355)
		Canadian (C. I. 1625)	Gothland (C. I. 1898)	Monarch (C. I. 1876)	Black Mesdag (C. I. 1877)	Richland (C. I. 787)	Fulghum (C. I. 708)	
<i>Ustilago levis</i> parent.....		100	0		0			7
Buff mutant race.....	1	84	0	0	0	17	0	0
<i>U. avenae</i> parent.....		98	77	6	0	0	0	0
Buff mutant parent.....		84	0	0	0	17	0	0
Buff hybrid race.....	2	85	58	0	0	3	0	0
Buff field race.....	3	87	66	1	0	29	3	0
<i>U. levis</i> parent.....		100	0	24	83	0	0	0
Buff mutant parent.....		84	0	0	0	17	0	0
Buff hybrid race.....	4	82	0	2	75	0	0	0
<i>U. levis</i> parent.....		100	0	24	83	0	0	0
Buff hybrid parent.....		85	58	0	0	3	0	0
Buff hybrid race.....	5	92	39	91	25	0	1	0
<i>U. levis</i> parent.....		100	0	67	0	34	0	0
Buff mutant parent.....		84	0	0	0	17	0	0
Buff hybrid race.....	6	80	0	21	0	20	0	55
<i>U. levis</i> parent.....		87	0	72	33	0	79	0
Buff mutant parent.....		84	0	0	0	17	0	0
Buff hybrid race.....	7	83	0	94	13	0	54	0

It is apparent from the foregoing results that new races of the buff smut may be produced at will by crossing any race of this fungus with races of *Ustilago avenae* and *U. levis*. A new race of the buff smut was obtained from every cross made between races of the buff smut and different races of these two species. In some cases the new races are similar in pathogenicity to the *U. avenae* or *U. levis* parent, while in others the virulence of the hybrid race is greater than that of either parent. For example, race 2 and its *U. avenae* parent both infect Gothland while all other varieties are resistant, and race 7 and its *U. levis* parent both infect Monarch, Black Mesdag, and Fulghum. Race 4, however, infects Black Mesdag only, whereas the *U. levis* parent infects Monarch in addition to Black Mesdag. On the other hand, race 5 infects all of the varieties (Gothland, Monarch, Black Mesdag) that the two parent races infect; and race 6, like its *U. levis* parent, infects Monarch and Richland but, unlike either parent, can infect Alabama Red Rustproof. Just how the infective capacity of race 6 for Alabama Red Rustproof arose cannot be explained by the data at hand.

Race 1 infects Canadian and, to some extent, Richland, and apparently it has the same pathogenicity as the *Ustilago levis* race from which it mutated. It is notable, however, that in earlier tests Monarch also was infected by this race (5). It would appear, therefore, that buff race 1 originally was heterozygous for pathogenicity and those biotypes capable of infecting Monarch were lost by selective elimination on another variety. Similar results have been obtained with race 2. Gothland and Monarch were infected by this race in the  $F_3$  (5), whereas only Gothland was infected in the  $F_8$  (table 1). Sampson and Western (9) have shown that there is a definite selective influence of the host on the relative stability of physiologic races of the oat smut fungi. They pointed out, however, that it would be theoretically possible for a heterozygous condition to persist through several chlamydospore generations, which would limit the efficiency of screening as a means of obtaining races pure for patho-

genicity. Possibly this was the case with race 2, and the repeated selection of inoculum from Gothland was necessary to gradually eliminate the capacity for infecting Monarch. Little is known, however, regarding the stability of such hybrid races of the oat smuts. The majority of the races listed in table 1 (races 4, 5, 6, and 7) are in the  $F_4$  generation, and if inoculum of future generations is taken from the same variety then the pathogenicity for certain other varieties may become lost. This would be highly probable in the case of a hybrid race that carries the pathogenic properties of both parents, such as race 5 (table 1). Race 5 was obtained by selecting a buff  $F_2$  segregate on Monarch, and inoculum has been taken from the same variety in succeeding generations. Repeated selection of inoculum of this race from Monarch, however, might finally eliminate the Gothland and Black Mesdag pathogenicity, or the selection of inoculum from Gothland might result in the loss of the Monarch and Black Mesdag virulence. On the other hand, if there were linkage of factors for virulence on these varieties, or if this virulence were controlled by a single factor, both of which considerations seem improbable in this case, then the reaction would remain constant in future generations regardless of the variety from which inoculum might be obtained, unless the linkage were to become broken or mutation were to occur.

Similar results might be expected with races 6 and 7, while race 4 probably will remain constant in virulence if the inoculum is always taken from Black Mesdag. Therefore, in the light of these considerations, it is possible that the host reaction of the buff smut races shown in table 1 will change to some extent by continuous propagation on specific varieties. Considering the pedigree of these races, however, it seems improbable that such changes as may occur will materially affect their differentiation.

It will be noted in table 1 that comparative tests with the parent or parents of race 3 were not made, the reason being that its pedigree is not known. This race was discovered in 1936 among a large number of collections of *Ustilago avenae* and *U. levis* obtained from the 1935 seed stock nursery at Aberdeen, Idaho. The row from which this buff smut specimen was obtained had been sown to seed collected in a grain elevator in Chicago, Ill. Eleven smutted panicles were in the collection, of which four were *U. avenae*, four *U. levis*, and three buff smut. No other specimens of buff smut were found in 1,877 smutted panicles examined, all of which came from the seed stock nursery mentioned above. This is the only record of the occurrence of the buff smut under natural conditions. Furthermore, the record would seem to preclude any possibility that this instance of natural occurrence of the buff smut was due to an escape from the experimental material, especially in view of the fact that the panicles smutted with race 3 are characteristically different from those of race 1, the original buff smut, or any of the hybrid races. Race 1 produces sori entirely covered by the outer glumes, while race 3 completely destroys the outer glumes, leaving the sori exposed. The other races, for the most part, partially destroy the outer glumes, thus producing a type of smutted panicle intermediate between the two extremes of races 1 and 3. The spores of the buff smut, like

those of *U. levis*, remain intact in the sorus and are not readily disseminated as in *U. avenae*. Since the original buff smut race arose by mutation (5) it seems probable that race 3 is the result of a recurring mutation in *U. levis* that took place under natural conditions. Consequently, the buff smut fungus might justifiably be recognized as a variety of *U. levis* or possibly even as a distinct species.

#### CYTOLOGY

The results of studies on nuclear behavior in the buff smut fungus indicate that this phenomenon is fundamentally the same as in *Ustilago avenae* and *U. levis*. The nuclear condition in the various stages of development that were observed is shown in figure 1. The mature chlamydospore contains a single diploid nucleus (fig. 1, A). Meiotic division of this nucleus accompanies spore germination and one nucleus becomes located in each cell of the promycelium (B). The promycelium occasionally has four cells but usually three, the spore functioning as the fourth cell. Mitotic division of the nuclei of the promycelium accompanies the budding of a sporidium from each cell and one nucleus passes into each sporidium, the other remaining in the promycelial cell from which other sporidia may bud (C). Each sporidium contains a single haploid nucleus, which divides when the sporidium buds and one nucleus passes into the daughter sporidium. Binucleate sporidia frequently are observed, in which case it is presumed that nuclear division preceded sporidial budding (D). When sporidia of opposite sex fuse, the nucleus from one passes through the copulation tube into the other, thus initiating the dikaryophase (E). Each pair of fused sporidia produces an infection hypha into which the paired nuclei pass (F). Presumably the binucleate condition persists throughout the parasitic stage, nuclear fusion occurring when the spores become mature. If this presumption is correct, then nuclear behavior in the buff smut is identical with that of *U. avenae* and *U. levis*.

#### INHERITANCE OF SORUS TYPE

Two types of smutted panicles produced by two races of *Ustilago avenae* were observed in one row of Victory (C. I. 560) oats in the physiologic race nursery at Pullman, Wash., in 1937. Most of the smutted panicles in this row had the usual brown powdery type of sorus. A few panicles, however, had a distinctly black indurate type of sorus, and the spores of this type were slightly darker in color and less prominently echinulate than those of the powdery type. In both types the glumes had been completely destroyed, as shown in figure 2. The results of inoculations with spores from the two types of sorus indicated that these characters were genetically distinct. Studies were undertaken, therefore, to determine the nature of their inheritance. Combinations of monosporidial lines were used for inoculum, and seed of Anthony (C. I. 2143) oats was inoculated and grown to maturity in the greenhouse for the  $F_1$  and in the field for the  $F_2$ . The results of these studies are summarized in tables 2, 3, and 4.



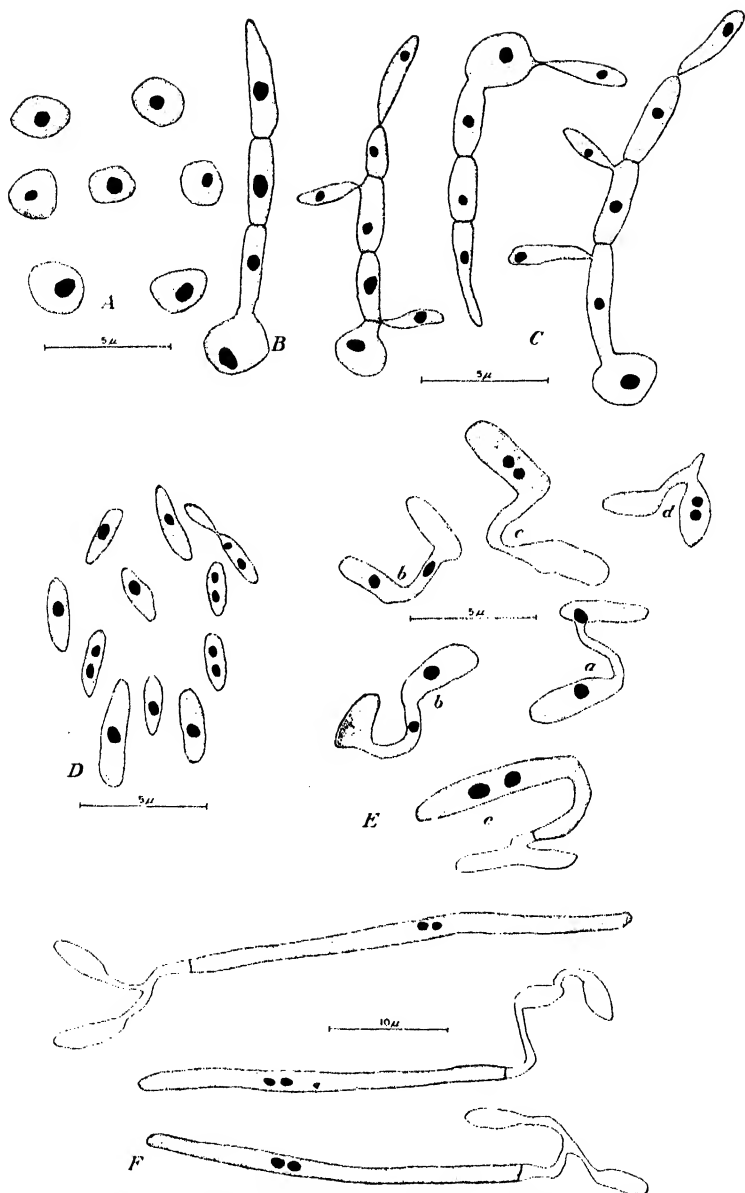


FIGURE 1.—Camera-lucida drawings showing the nuclear condition in various stages of development of the buff smut fungus. *A*, Mature chlamydospores. *B*, Germinated chlamydospore prior to sporidial formation. *C*, Germinated chlamydospores with sporidia budding from the promycelial cells. *D*, Haploid uninucleate and binucleate sporidia. *E*, Pairs of conjugated sporidia showing nuclei in various stages of migration: *a*, Nucleus about to enter the fusion tube; *b*, nucleus passing through the tube; *c*, binucleate stage or dikaryophase resulting from the migration of the nucleus from one sporidium to the other; *d*, beginning the production of the infection hypha following initiation of the dikaryophase; *e*, binucleate stage in which nuclear migration was accompanied or followed by migration of the cytoplasm into the sporidium which contains the nuclei. *F*, Infection hyphae.



FIGURE 2.—Smutted panicles of Victory oats showing two types of sorus produced by two physiologic races of *Ustilago avenae*. A, Powdery type, which sheds spores readily. (Note spore masses that were shed when the panicles were tapped against the background.) B, Indurate type, which does not shed spores.

TABLE 2.—Type of sorus produced on Anthony oats by two races of *Ustilago avenae* and hybrids between them

Parent sorus type	Chlamydo-spore No.	Number of sporidial combinations	Type of sorus produced by—	
			F <sub>1</sub>	F <sub>2</sub>
Powdery.....	56	4	Powdery ...	Powdery.
	57	4		
	58	4		
	54	4		
Indurate.....	55	4	Indurate....	Indurate.
	59	2		
	60	2		
	56×54	8		
Powdery and indurate.....	57×55	8	Powdery ...	Powdery and indurate.
	58×59	6		
	58×60	6		
		6		

TABLE 3.—Segregation of factors <sup>1</sup> for type of sorus in crosses between two physiologic races of *Ustilago avenae*

F <sub>1</sub>				F <sub>2</sub>		
Chlamydospore No. and genotype	Sporidium No.	Sex <sup>2</sup>	Gametes	Geno-type	Ratio	Type of sorus
93 ( <i>Pp</i> )	1	+	<i>p</i>	<i>PP</i>	1:2:1	Powdery.
	2	+	<i>P</i>	<i>Pp</i>		Do.
	3	—	<i>P</i>	<i>Pp</i>		Do.
	4	—	<i>p</i>	<i>pp</i>		Indurate.
94 ( <i>Pp</i> )	1	+	<i>P</i>	<i>Pp</i>	4:0	Powdery.
	2	—	<i>p</i>	<i>Pp</i>		Do.
	3	+	<i>P</i>	<i>Pp</i>		Do.
	4	—	<i>p</i>	<i>Pp</i>		Do.
95 ( <i>Pp</i> )	1	+	<i>p</i>	<i>PP</i>	1:2:1	Powdery.
	2	—	<i>P</i>	<i>Pp</i>		Do.
	3	+	<i>P</i>	<i>Pp</i>		Do.
	4	—	<i>p</i>	<i>pp</i>		Indurate.
96 ( <i>Pp</i> )	1	+	<i>P</i>	<i>PP</i>	1:2:1	Powdery.
	2	—	<i>p</i>	<i>Pp</i>		Do.
	3	—	<i>p</i>	<i>Pp</i>		Do.
	4	—	<i>p</i>	<i>pp</i>		Indurate.
97 ( <i>Pp</i> )	1	+	<i>P</i>	<i>Pp</i>	1:0	Powdery.
	2	—	<i>p</i>	<i>Pp</i>		Do.
	3	—	<i>p</i>	<i>Pp</i>		Do.
	4	—	<i>p</i>	<i>Pp</i>		Do.

<sup>1</sup> *P*=powdery; *p*=indurate.  
<sup>2</sup> Plus (+) and minus (—) signs indicate sporidia of opposite sex.

TABLE 4.—Results obtained by backcrossing sporidia from a hybrid chlamydospore with sporidia from the parent chlamydospores to determine the heritability of sorus type

USTILAGO AVENAE 94 (*Pp*) × U. AVENAE 56 (*PP*)

U. avenae 94 ( <i>Pp</i> ) hybrid			U. avenae 56 ( <i>PP</i> ) parent			Backcross	
Sporidium	Sex	Gamete	Sporidium	Sex	Gamete	Geno-type	Type of sorus
1	+	<i>P</i>	2	—	<i>P</i>	<i>PP</i>	Powdery.
1	+	<i>P</i>	3	—	<i>P</i>	<i>PP</i>	Do.
2	—	<i>p</i>	1	+	<i>P</i>	<i>Pp</i>	Do.
2	—	<i>p</i>	4	+	<i>P</i>	<i>Pp</i>	Do.
3	+	<i>P</i>	2	—	<i>P</i>	<i>PP</i>	Do.
3	+	<i>P</i>	3	—	<i>P</i>	<i>PP</i>	Do.
4	—	<i>p</i>	1	+	<i>P</i>	<i>Pp</i>	Do.
4	—	<i>p</i>	4	+	<i>P</i>	<i>Pp</i>	Do.

USTILAGO AVENAE 94 (*Pp*) × U. AVENAE 54 (*Pp*)

U. avenae 94 ( <i>Pp</i> ) hybrid			U. avenae 54 ( <i>pp</i> ) parent			Backcross	
Sporidium	Sex	Gamete	Sporidium	Sex	Gamete	Geno-type	Type of sorus
1	+	<i>P</i>	2	—	<i>p</i>	<i>Pp</i>	Powdery.
1	+	<i>P</i>	3	—	<i>p</i>	<i>Pp</i>	Do.
2	—	<i>p</i>	1	+	<i>p</i>	<i>pp</i>	No infection.
2	—	<i>p</i>	4	+	<i>p</i>	<i>pp</i>	Indurate.
3	+	<i>P</i>	2	—	<i>p</i>	<i>Pp</i>	Powdery.
3	+	<i>P</i>	3	—	<i>p</i>	<i>Pp</i>	Do.
4	—	<i>p</i>	1	+	<i>p</i>	<i>pp</i>	No infection.
4	—	<i>p</i>	4	+	<i>p</i>	<i>pp</i>	Indurate.

As shown in table 2, the 12 monosporidial combinations representing three chlamydospores from the powdery type of sorus produced that type of sorus in the F<sub>1</sub> and F<sub>2</sub>, and the 12 monosporidial combi-

nations representing four chlamydospores from the indurate type produced that type of sorus. Apparently, therefore, the chlamydospores from which the monosporidial lines were obtained were homozygous for sorus type. The 28 combinations between monosporidial lines from the two types of sorus produced powdery sori in the  $F_1$ , indicating dominance of the factor for this type of sorus. In the  $F_2$ , there was segregation into powdery and indurate sorus types. The manner in which segregation of factors for the two types of sorus occurs is shown by the results from inoculations with crosses between monosporidial lines obtained from five  $F_1$  chlamydospores (table 3), and by backcrossing the monosporidial lines from one  $F_1$  spore with four monosporidial lines from one chlamydospore of each parent (table 4). As shown in table 3, there was independent segregation of factors for sex and sorus type in three of the chlamydospores (93, 95, 96), and the sorus type appeared in a ratio of 3 powdery to 1 indurate. The genotype ratio was found to be 1:2:1, as indicated. In chlamydospores 94 and 97, segregation of factors for sex and sorus type occurred in the same nuclear division and, therefore, all of the sori were powdery, the ratio being 4:0, as shown. Theoretically, these  $F_2$  chlamydospores are heterozygous and should produce both types of sorus in a 3:1 ratio in the  $F_3$ , if segregation of factors for sex and sorus type occurs independently. In the backcrosses with the powdery parent (*Ustilago avenae* 56, table 4) all of the combinations produced powdery sori, as expected, since this character is dominant. The spores of half of these backcrosses were determined to be homozygous for the powdery sorus and the other half heterozygous. In the backcrosses with the indurate parent, four of the crosses produced powdery sori the spores of which were heterozygous, two crosses produced indurate sori the spores of which were homozygous, and two crosses failed to infect. Presumably the two crosses that failed to infect would have produced the indurate type of sorus if infection had occurred (table 4), in which case the spores would have been homozygous. All of the indurate sori were black, in contrast to the dark brown of the powdery sori. This color contrast probably is due to the slightly darker spores in conjunction with color intensification caused by a more effective elimination of air spaces in the indurate sorus.

It is apparent from these studies that the factor for powdery sorus type is dominant over the factor for indurate sorus type and that the segregation and recombination of factors in the  $F_2$  occurs in a 1:2:1 ratio. Chlamydospore markings and color are known to be inherited in a similar manner (6).

#### SPORIDIAL FUSIONS

In a previous report (4) it was shown that in the fusion process between any two sporidia of *Ustilago avenae* and *U. levis* one sporidium is active while the other appears to be passive. Since the sporidia normally are morphologically indistinguishable it was not determined whether the active sporidia were of the same sex and the passive sporidia of the opposite sex. Recently, however, it was found possible to modify the morphology of sporidia to such an extent that those of one line can readily be distinguished from those of another line. As shown in figure 3, sporidia that bud on potato-dextrose agar are larger and more uniform in size and shape (fig. 3, A, D) than those

that bud on plain agar (fig. 3, *B*, *E*). Furthermore, sporidia from plain agar usually have no food vacuoles, while those from potato-dextrose agar may have one to several, the usual number being two.

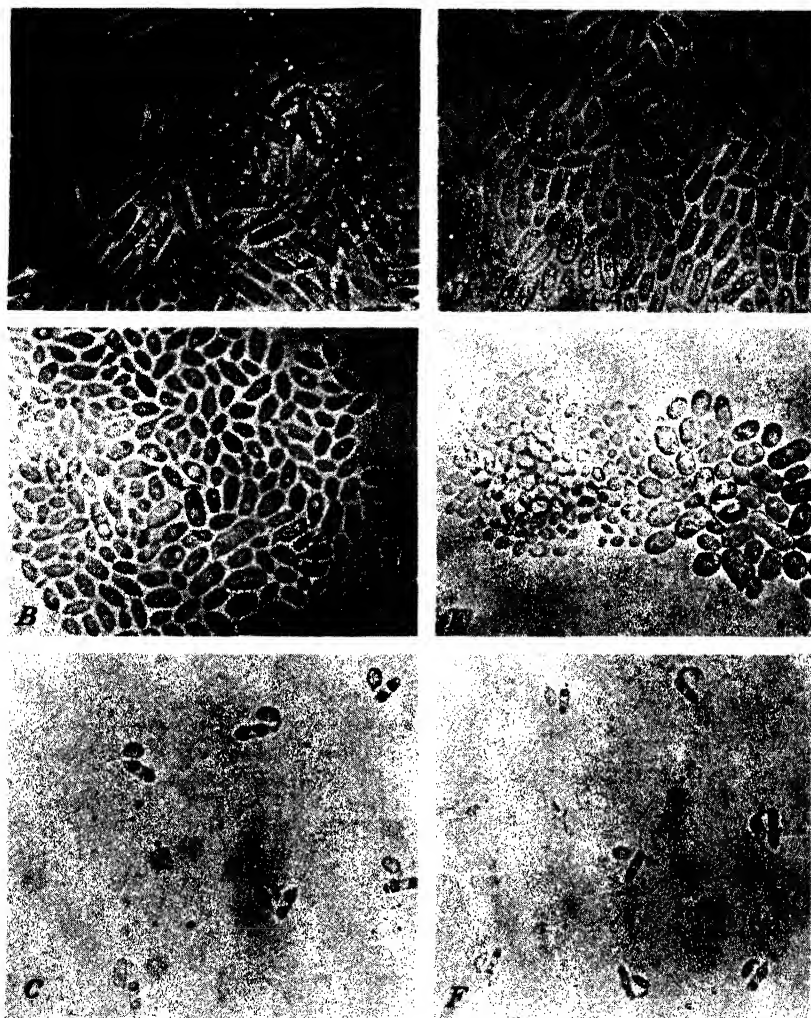


FIGURE 3.—Photomicrograph of *Ustilago levis* and buff smut sporidia showing differences in size and shape on potato-dextrose agar and plain agar and pairs of sporidia in various stages of fusion. *A*, *U. levis* 91-1 on potato-dextrose agar; *B*, on plain agar; *C*, pairs of *U. levis* sporidia from the two media, showing stages of the fusion process; *D*, buff 55-4 on potato-dextrose agar; *E*, on plain agar; *F*, pairs of the buff smut sporidia from the two media showing stages of the fusion process.

Thus, for example, by pairing the sporidia of sex A grown on potato-dextrose agar with the sporidia of sex B grown on plain agar, or the sporidia of sex A grown on plain agar with the sporidia of sex B grown

on potato-dextrose agar, it is possible to determine which sporidia are active and which are passive in the fusion process.

Such pairings, which may be made with the aid of a micromanipulator, are shown in figure 3, *C*, *F*, and the ease with which the sporidia are distinguished from each other is obvious. By this means observations were made on pairs of plus (+) and minus (−) sporidia of *Ustilago avenae*, *U. levis*, and the buff smut. In 49 pairs of *U. avenae* sporidia, the plus (+) sporidia, taken from plain agar, were active and the minus (−) sporidia, taken from potato-dextrose agar, were passive, while in 39 other pairs the minus (−) sporidia, taken from plain agar, were active and the plus (+) sporidia, taken from potato-dextrose agar, were passive. In 10 pairs, however, the plus (+) sporidia, taken from potato-dextrose agar or plain agar, were active while the minus (−) sporidia, taken from either medium, were passive. Thus, in 88 pairs the sporidia taken from plain agar were active and the sporidia taken from potato-dextrose agar were passive, regardless of sex, while in 10 pairs the plus (+) sporidia were active and the minus (−) sporidia were passive, regardless of the medium from which they were taken. In 93 pairs of *U. levis* sporidia and 80 pairs of buff smut sporidia the sporidia taken from plain agar were active and those taken from potato-dextrose agar were passive, regardless of sex. Therefore, it appears from these observations that apparent active or passive participation in the fusion process is governed primarily by the "physical" condition of the sporidia and not by their sex. Obviously, those sporidia which are grown on plain agar are in a "starved" condition and apparently respond more rapidly to the fusion stimulus than those which are grown on potato-dextrose agar and have an abundance of reserve nutrients. The 10 pairs of *U. avenae* sporidia mentioned above were an exception to the general rule.

The increased tendency for sporidia of the smut fungi to fuse when placed under conditions of low nutrients is a generally recognized fact. Consequently, the mating of sporidia from a low-nutrient medium with sporidia from a high-nutrient medium might be considered an unfair test of their active and passive reactions. For this reason it seemed desirable to mate sporidia of opposite sex that had been grown on the same medium. Accordingly, observations were made on pairs of sporidia in which the larger sporidium of each pair represented one sex and the smaller sporidium the other sex, both sporidia being taken from potato-dextrose agar. Twenty pairs of buff smut sporidia were observed and the plus (+) sporidium was active in 9 pairs, the minus (−) sporidium was active in 9 pairs, and both sporidia were active in 2 pairs. Similar results were obtained with 20 pairs of *Ustilago avenae* sporidia, but no observations were made on *U. levis*. These results further indicate that sporidia of opposite sex in the oat smut fungi may appear active or passive in the fusion process, depending, at least in part, upon their "physical" condition.

In the course of the studies on sporidial fusions, two sizes of sporidia were observed in one monosporidial line of the buff smut on plain agar. This size difference can be seen in figure 3, *E*, where a colony of the larger sporidia is shown adjacent to and coalesced with a colony of the smaller sporidia. These two colonies developed from single

balerna to the tobacco mosaic virus. The strain of Ambalema selected by the senior writer and used in extensive breeding studies appears to be one which is practically immune. When it is inoculated in the field with a nonnecrotic spotting (?) white mosaic virus, the local lesions develop prominently in the course of a few days as bright yellow spots which attain a diameter of 5 mm. or less. The virus appears to be completely localized in these, for no further chlorotic spots develop on the plants. That there is no barrier to rapid long-distance movement of virus in highly mosaic-resistant plants was proved by Valteau (?) when he demonstrated long-distance spread of the necrotic-spotting white mosaic virus in Ambalema which is practically immune to it. The virus multiplied in a Turkish tobacco scion grafted to Ambalema, flowed from the scion to developing leaves of Ambalema where it caused numerous scattered necrotic spots (from which the virus was recovered), and caused no further symptoms.

Hybrids of burley tobacco with Ambalema produce plants in the  $F_2$  generation with all degrees of resistance between that of Ambalema and susceptible burley. The resistant plants in the  $F_2$  generation are those in which the yellow or white strains of the virus appear to be completely localized in yellow spots; those in which there is delayed movement of a small amount of virus from local lesions to leaves higher on the plant, where it multiplies very slowly and causes yellow spots; those in which larger quantities of virus move into developing leaves, multiply moderately rapidly to produce ring patterns (pl. 2) but do not invade the growing point; and possibly plants of a lesser degree of resistance which are difficult to distinguish from susceptible plants except that the young leaves are mottled but not distorted. In the present study numerous mosaic-resistant burley and dark tobacco plants have been under observation both in the greenhouse and in the field during several years, but the exact distribution of virus was studied in hybrid burley plants in which local yellow spots developed when inoculated with "pure white" mosaic, and distinct yellow spots developed at points to which virus was carried higher on the plant. It is the object of this paper to report these findings and to discuss their application to pattern development in susceptible plants.

#### DISTRIBUTION OF VIRUS IN MOSAIC-RESISTANT PLANTS

The exact distribution of the "pure white" mosaic virus in a resistant burley plant was studied in an  $F_2$  ( $F_2$  burley  $\times$  Ambalema)  $\times$  burley. A lower leaf (leaf 2, fig. 1) was inoculated heavily. The initial spots finally coalesced to form a large yellow area. Virus was carried from this area to leaves higher on the plant where the individual particles or aggregates were distributed, multiplied, and again caused yellow spots. Invasion was largely confined to one sector of the plant as leaves on the opposite side remained free or nearly free from yellow spots.

The extent of invasion of the plant as indicated by yellow patterns is represented in black in figure 1. From the points marked *g* green disks were cut, and from the points marked *y* yellow disks were cut with a cork borer 0.4 cm. in diameter. Six disks were cut from each

leaf. The borer was sterilized in boiling water before each cut. Each disk was ground separately in 2 drops of  $\frac{M}{10}$  disodium phosphate and rubbed on a portion of a leaf of a burley test plant containing the necrotic spotting factor from *Nicotiana glutinosa*. The necrotic spots

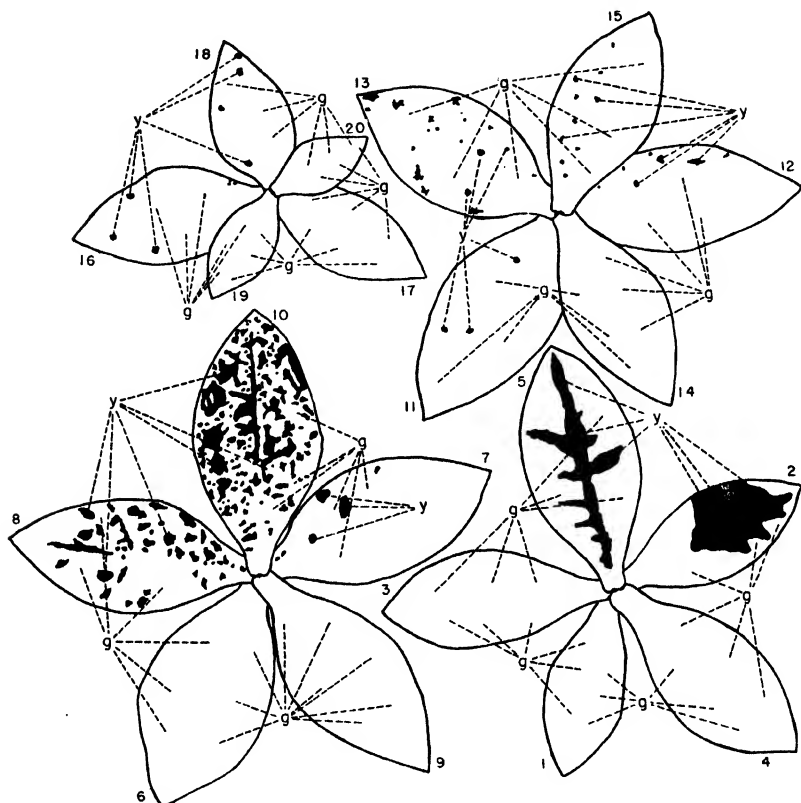


FIGURE 1. Distribution of chlorotic patterns (black) in all the leaves of a burley plant, containing the mosaic-resistant factors from Ambalema, following inoculation with white mosaic on leaf 2. *g* indicates points from which green disks of tissue were cut and tested on a necrotic-spotting burley plant; all green disks proved to be virus-free. *y* indicates points from which yellow disks were cut all of which contained virus.

were counted 8 days later. The results of the inoculations are shown in table 1.

Disks from leaves without symptoms caused no necrotic lesions in the test plants. Green disks from all patterned leaves proved to be free from virus in spite of extensive invasion of some of these leaves and the close proximity of the green tissue to chlorotic viruliferous tissue. (See leaf 10, fig. 1.) All yellow disks proved to be from invaded areas as expected and contained a fairly high concentration of virus. Localization of virus in the yellow areas is clearly brought out. The results of this study demonstrate that in both old and young leaves of plants inoculated with a bleaching strain of the tobacco



mosaic virus, the virus causes yellow spots wherever it multiplies in leaf tissue and its distribution within the leaf is quite accurately indicated by the distribution of chlorotic spots.

TABLE 1.—*Virus concentration in yellow or white areas in a resistant burley plant as shown by the number of necrotic spots produced when macerated tissue from these areas was rubbed onto leaves of burley plants containing the N factor from Nicotiana glutinosa*

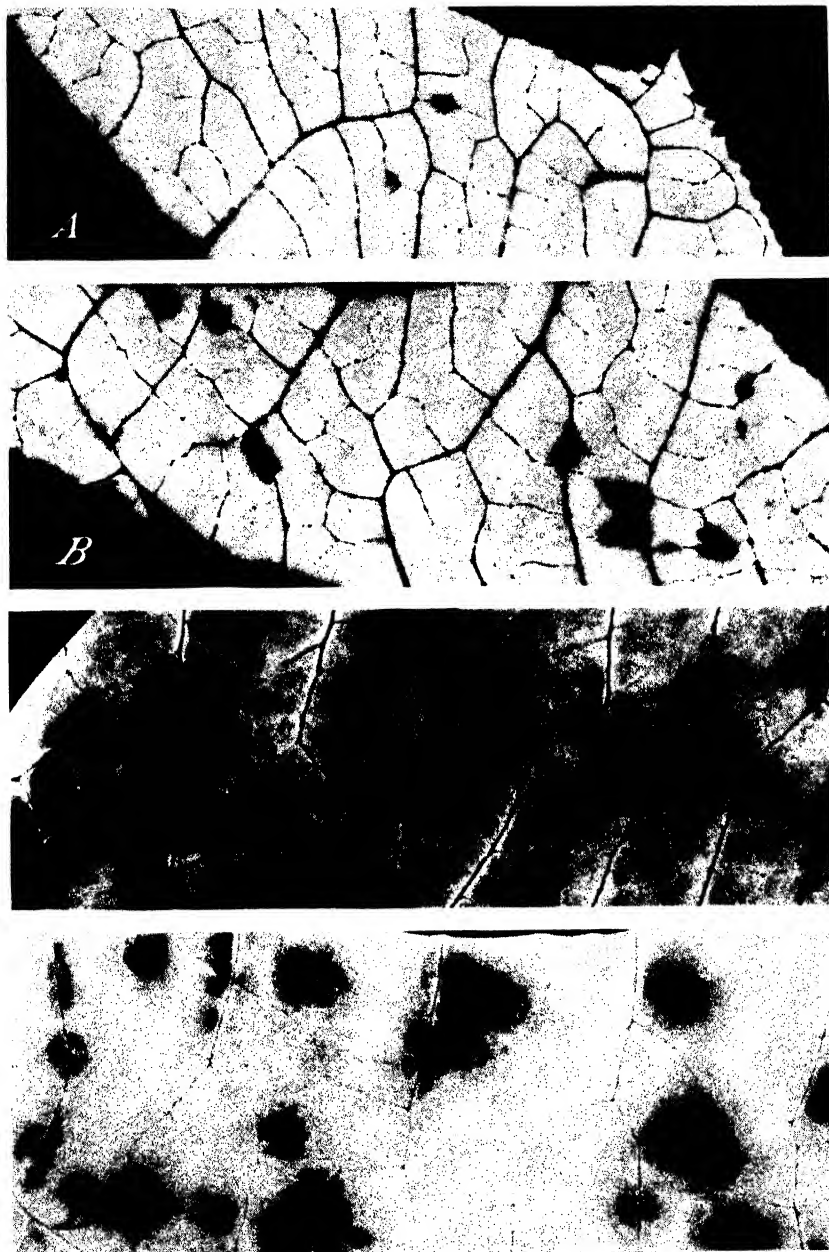
[Leaves 1, 3, 4, 6, 9, 14, 17, 19, 20, and 21 were symptomless]

Leaf No. (numbered from bottom)	Number of necrotic spots <sup>1</sup> produced by disks from yellow or white areas (y)			Leaf No. (numbered from bottom)	Number of necrotic spots <sup>1</sup> produced by disks from yellow or white areas (y)		
	Disk No. 4	Disk No. 5	Disk No. 6		Disk No. 4	Disk No. 5	Disk No. 6
1				10	8	29	9
2	22	15	14	11	13	9	5
3				12	18	6	7
4				13	1	9	2
5	14	24	19	14			
6				15	10	4	4
7	18	24	10	16	15	11	9
8	26	14	7	17			
9				18	23	9	6

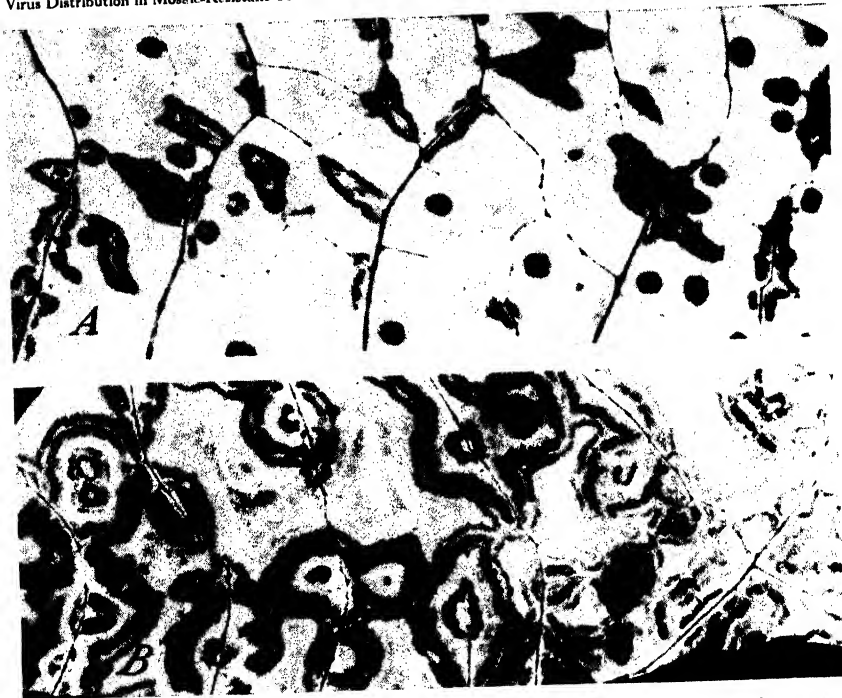
<sup>1</sup> Disks from leaves without symptoms and from green areas of affected leaves produced no necrotic spots on the test plants.

Another degree of resistance of a burley plant carrying two factor pairs for resistance from Ambalema is illustrated in plate 1. The photographs reproduced were contact prints made from leaves at various distances from the growing point of a mosaic-resistant burley plant. They are reproduced in natural size; *A*, leaf 32 is near the top of a large plant just developing a blossom bud. *B* is leaf 30; *C*, leaf 29, and *D*, leaf 24. The portion of leaf photographed is that between two secondary veins.

In leaf 32 (*A*) a few single virus particles or aggregates have been carried to points on small veins where they have multiplied, spread from cell to cell, and produced chlorotic (black on the contact print) spots. In leaf 30 (*B*) the spots are larger and somewhat more numerous. In the older leaf 29 (*C*) the spots have increased in size and are beginning to coalesce, which indicates that the tissues are slowly becoming solidly invaded. In leaf 24 (*D*) definite ring patterns are evident, and certain portions of the leaf are completely invaded. In plate 2, *A* and *B* are from another plant of the same degree of resistance; *A* represents a recently invaded leaf showing numerous centers from which the virus is slowly spreading by cell-to-cell movement; and *B* represents a leaf which has been slowly but completely invaded. The ring-spot symptom in this and other virus diseases is an indication that invasion occurred when the leaves were well developed. From the practical standpoint the degree of resistance shown in figure 1 represents immunity in the field, while the degree of resistance shown in plates 1 and 2 is such that with moderately heavy infection at setting time with a rapidly multiplying bleaching strain of virus the plants will become slowly invaded and infection will consist in scattered chlorotic ring patterns but with no distortion and only a slight retardation of growth. Inoculation after the plants have commenced rapid growth usually causes only local infections. Under farm conditions where a susceptible variety becomes heavily infected plants of the degree of resistance illustrated in plates 1 and 2 show no evidence of infection.



Invasion by white mosaic virus of a White Burley tobacco plant containing the two factor pairs from Ambalema: *A*, Leaf 32; *B*, leaf 30; *C*, leaf 29; *D*, leaf 24. Leaf 32 is from near the top of the plant; the others are numbered in descending order on the stalk. Dark areas represent chlorotic spots where virus particles have been carried through the vascular system, become lodged, and have multiplied. Contact prints made by using a portion of the leaf between two secondary veins as the "negative." Natural size.



Invasion by white mosaic virus of a White Burley tobacco plant of the same degree of resistance as the plant shown in plate 1: *A*, Many of the centers of multiplication of the virus are indicated by the black (white or light yellow) spots and circles, the tissue having been almost completely invaded but not yet bleached; *B*, complete invasion of the leaf tissue by cell-to-cell spread of the virus from numerous centers, the ring patterns having developed after the leaf was fully grown. Contact prints shown. Natural size.

VIRUS DISTRIBUTION AS RELATED TO PATTERN DEVELOPMENT  
IN SUSCEPTIBLE TOBACCO

In addition to the practical value of the use of white mosaic virus in breeding for resistance the results are of theoretical value in giving a better understanding of pattern development in susceptible plants. The tobacco mosaic virus produces several distinct sets of symptoms in tobacco plants, depending upon the strain of the virus used and the genotype inoculated. Routine breeding studies for mosaic resistance and the results of the present studies have thrown much light on the nature of these patterns, a discussion of which may tend to give those unfamiliar with mosaics a better understanding of pattern development.

The development of the ordinary green patterns produced by most of the common strains of the tobacco mosaic virus can be understood if one realizes what occurs in a slowly invaded mosaic-resistant plant. The leaves illustrated in plate 1 were well developed before virus particles invaded them. The particles were scattered through the leaves and each formed the center of a chlorotic pattern. If the growing point of this resistant plant together with the virus present were telescoped to form the growing point of a susceptible diseased plant then the development of patterns in the susceptible variety would be readily understood. The youngest leaf primordia would be virus-free, formed leaves in the bud would contain an occasional virus particle, slightly older leaves would have chlorotic areas or patterns where particles had multiplied from a few centers, and still older leaves would be patterned but would be invaded more or less solidly because of cell-to-cell invasion into healthy tissue surrounding the points of virus multiplication. A limited study of virus distribution in growing-point leaves has indicated that invasion of growing points of susceptible plants takes place in the manner described and that much of the tissue of the growing point is virus-free.

Certain of the ordinary green strains of tobacco mosaic virus cause the inoculated area on a fully grown leaf to slowly become chlorotic and then necrotic. The virus carried to leaves too old to develop patterns but young enough to be expanding rapidly, quickly invades them from numerous centers where particles are deposited, and finally causes extensive necrosis. These are the burning strains. In the younger leaves ordinary mottle patterns with but little necrosis develop.

Ring patterns are a common symptom of virus diseases (3). Occasionally a plant is found affected by a strain of tobacco mosaic which causes ring patterns and which produces them when transferred to other tobacco plants and to tomatoes (2). Plates 1 and 2 show that ring patterns develop when virus particles are distributed in fairly well-developed leaves too late to prevent the initial normal development of chloroplasts. The bleaching strains, as they advance from these centers into moderately resistant tissue, develop the "Liesegang" type of pattern made up of bands of green and yellow tissue (pl. 2.) The leaf finally bleaches to a light green or yellow. Any yellowing strain which multiplies at only a moderate rate could cause ring patterns in a susceptible variety because chloroplast development would proceed faster than virus multiplication and spread in young leaves, and the leaves would attain considerable size with no distor-

tion before the infected spots became apparent. As the virus invaded the older and more resistant tissue, ring patterns would result as in the mosaic-resistant leaves pictured in plate 2.

The patterns shown in figure 1 are typical of speckled mosaic, a disease which is of rather rare occurrence in nature (4). A slowly multiplying bleaching virus, rather than a slow-moving one, as postulated by Norval (6), can produce exactly the same effect in a susceptible plant as a rapidly multiplying one in a highly resistant plant. In each case the virus remains in the inoculated area an abnormally long time, and when released is carried to leaves higher on the plant where it again multiplies so slowly that only yellow specks develop as the leaves expand. Thus the growing-point tissue and young leaves in the bud are practically virus-free and distortion does not occur.

Masked strains such as Holmes (1) obtained by heat treatment have been collected by the senior writer on several occasions from solanaceous weeds collected at random in an old tobacco-growing area. On inoculated Turkish tobacco plants an occasional, faint, green ring pattern can be seen which may disappear in the course of a day or so. A masked strain seems to be in the nature of a non-bleaching, slow-multiplying strain. Young leaves are uninvaded or slowly invaded and visible patterns are not formed except for occasional faint rings.

#### SUMMARY

The progress of a bleaching strain of the tobacco mosaic virus can be followed accurately in a mosaic-resistant plant by the development of chlorotic patterns in invaded leaves. The virus appears to be confined almost completely to chlorotic areas, especially in old leaves. The distribution of chlorotic spots in a highly resistant plant inoculated with a bleaching strain gives further proof that virus distribution in a plant is by long-distance movement of virus particles or aggregates of particles, from points of inoculation and multiplication, followed by slow cell-to-cell movement of virus in the immediate vicinity of the point of lodgment of the virus particle. Resistance appears to result in slow multiplication of virus at the points of inoculation and slow release or complete failure of release of the virus into vascular tissue for long-distance spread. The mechanism which controls release of virus from local infections in susceptible and resistant varieties is not known.

Rate of multiplication of virus, rather than rate of actual movement of virus particles within the plant, seems to explain the difference between so-called slow-moving and rapidly moving virus strains. There appears to be no evidence that one strain moves more rapidly than another once it is released from local infections for long-distance transfer.

A study of virus distribution in mosaic-resistant plants aids in understanding pattern development in susceptible plants. Many of the different symptom complexes which occur in susceptible tobacco, such as mottled mosaic, ring mosaic, speckled mosaic, etc., which are correctly attributed to different strains of the virus, can be duplicated in resistant tobacco by the use of a single virus strain on plants of different degrees of resistance.

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# VIRUS DISTRIBUTION IN THE LEAVES OF MOSAIC-SUSCEPTIBLE TOBACCO PLANTS INOCULATED AT TOPPING TIME<sup>1</sup>

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## INTRODUCTION

The extent to which tobacco plants inoculated with mosaic virus at topping time may become invaded by harvesttime is not known. The movement of virus into well-developed uninoculated leaves of tomatoes and Turkish tobacco has been shown to be slow, 3 weeks being required for the invasion of rapidly growing tomato plants, with invasion still incomplete at the end of 3 months in field-grown fruiting tomato plants.<sup>2</sup>

This paper presents evidence on the distribution of virus in old leaves of susceptible plants of burley tobacco (*Nicotiana tabacum* L.) inoculated at topping time and its relation to visible symptoms when a bleaching strain of the virus is used.

## VIRUS DISTRIBUTION IN MATURING SUSCEPTIBLE WHITE BURLEY PLANTS

Three plants of Kentucky No. 16 burley growing in a ground bench in the greenhouse were topped and inoculated with the "pure white" mosaic virus, a strain isolated from white mosaic collected from pepper.<sup>3</sup> On plant 1, four immature top leaves were inoculated; on plant 2, three fully grown lower leaves were inoculated at three points equally distributed along the midveins; and on plant 3, all the leaves were inoculated at three points on one half of each leaf.

### UNINOCULATED LEAVES VIRUS-FREE 31 DAYS AFTER INOCULATION OF PLANT

After the inoculation of the plants the new growth developed white mosaic. The inoculated areas became yellow and gradually increased in size. The uninoculated leaves of plants 1 and 2 remained symptomless. Thirty-one days after inoculation, six cork-borer sections 1.5 cm. in diameter were cut from each uninoculated leaf of plants 1 and 2, from green portions of inoculated leaves of plants 2 and 3, and from infected suckers of all three plants. The six disks from a leaf were crushed together in 0.5 cc. of  $\frac{M}{10}$  disodium phosphate and rubbed on one-fourth of a leaf (25 to 30 cm. long) of a burley test plant carrying the necrotic spotting factor from *Nicotiana glutinosa*. The uninoculated leaves were all symptomless and proved to be virus-free (table 1). Apparently there had been no invasion of these leaves

<sup>1</sup> Received for publication April 15, 1940.

<sup>2</sup> BAWDEN, F. C. PLANT VIRUSES AND VIRUS DISEASES. 272 pp., illus. Leiden. 1939. See ch. 13.

<sup>3</sup> See JOHNSON, E. M. VIRUS DISEASES OF TOBACCO IN KENTUCKY. Ky. Agr. Expt. Sta. Bul. 306, pp. 285-415, illus. 1930.



TABLE 1.—*Number of necrotic spots produced on burley test plants when inoculated with disks cut from 2 Kentucky No. 16 burley tobacco plants 31 days after inoculation on top 4 and basal 3 leaves, respectively*

Plant No.	Part sampled	Number of disks	Necrotic spots produced
1.	(Leaves 1 to 11.....)	66	0
	Leaf 12 (inoculated).....	6	500
	Top suckers.....	6	400
	Basal suckers.....	6	500
	(Leaves 4 to 14.....)	66	0
2.	Leaves 1 to 3 (inoculated, green areas).....	18	0
	Basal suckers.....	6	400
	Top suckers.....	6	400

in the 31 days following inoculation. The inoculated leaves on plant 1 (upper) were spotted with numerous yellow spots and contained a high virus concentration. They appeared to be almost completely invaded. The mottled suckers, as would be expected, contained a high virus concentration. The uninoculated leaves of plant 2 and the green portions of the inoculated leaves (mature when inoculated) were symptomless and virus-free. The results indicate a rapid development of mosaic virus in young tissue (upper inoculated leaves of plant 1 and suckers) and an extremely slow cell-to-cell invasion of older leaf tissue (inoculated leaves of plant 2).

#### VIRUS INVASION OF UNINOCULATED LEAVES

Forty days after inoculation some of the middle leaves of plant 1 and the two upper leaves of plant 2 showed some chlorosis of the leaf-blade tissue along the bases of the midveins. After 45 days five leaves of plant 1 and the two upper leaves of plant 2 were chlorotic along the midvein and the secondary veins. After 52 days one more leaf on plant 1 was becoming chlorotic and after 59 days all but two leaves of plant 2 showed chlorosis along the base of the midrib (fig. 1). The invasion of these leaves was slow and evidently from cell to cell following the larger veins and slowly spreading from them into the blade tissue. From these results it is evident that the white mosaic virus can produce prominent symptoms in the invaded portion of old mature leaves.

#### VIRUS DISTRIBUTION IN INOCULATED LEAVES

After 31 days the inoculated leaves of plant 1 (four top leaves) were extensively invaded, as has been shown. The inoculated leaves of plant 2 (three bottom leaves) were mature at the time of inoculation and showed comparatively little invasion as indicated by chlorotic patterns. The leaves of plant 3 were in all stages of development from mature basal leaves to recently expanded top leaves when inoculated. Individual disks were cut from isolated green areas, from green areas near yellow areas, and from yellow areas of the inoculated leaves of plants 2 and 3. The disks were tested on burley test plants. The distribution of yellow patterns on the inoculated leaves, the location of the disks selected, and the results of inoculations with the disks are shown in figure 2, *A*, for plant 2 (31 days after inoculation) and in figure 2, *B*, for plant 3 (37 days after inoculation).

Figure 2, *A* and *B*, shows the slow development of chlorotic yellow patterns in old leaves inoculated more than 30 days previously, and

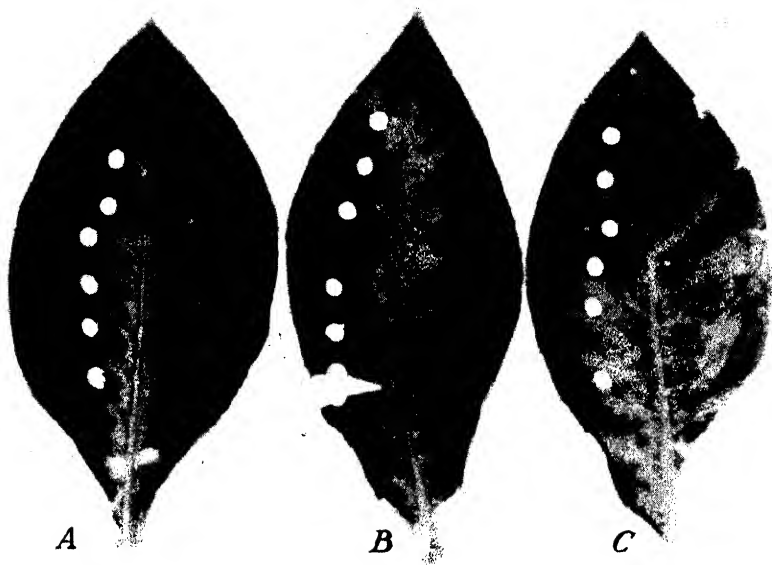


FIGURE 1.—Spread of pure white mosaic virus into old leaves of topped Kentucky No. 16 burley tobacco plant 1, which was inoculated at the top February 4, 1939. These leaves were symptomless until March 6, 1939. Photographed April 6, 1939. *A*, Eighth, *B*, sixth, and *C*, fifth leaf from bottom. Samples taken 31 days after inoculation were virus-free.

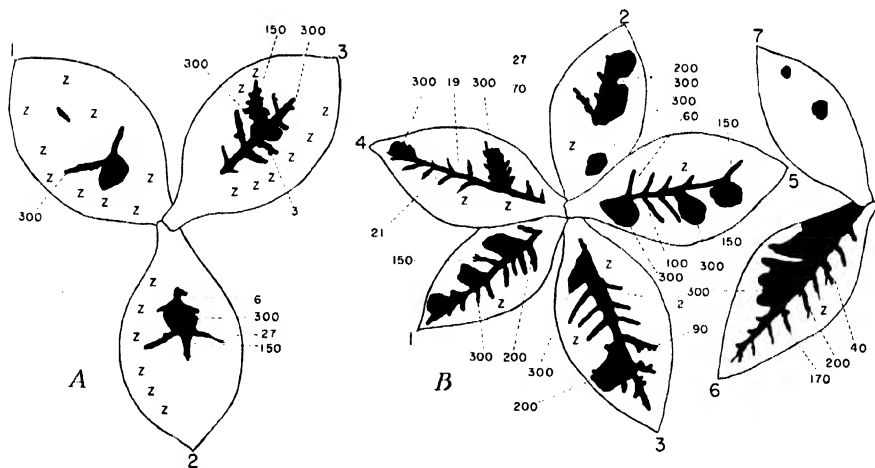


FIGURE 2.—*A*, Representation of chlorotic yellow patterns (black) on lower inoculated leaves 1, 2, and 3 of plant 2, 31 days after inoculation; *z* indicates points from which cork-borer sections were taken which produced no necrotic spots on test plants. The figures indicate the number of necrotic spots on test plants inoculated with sections taken at the end of the dotted lines. *B*, the same as *A* for plant 3, 37 days after inoculation. Leaves are numbered from below upward.

demonstrates that virus distribution is fairly accurately indicated by the yellow patterns in a susceptible plant. Yellow areas contained a high virus concentration, green areas located very near yellow areas were virus-free or contained a low virus concentration, while well-isolated green areas were virus-free. On plant 3 (fig. 2, *B*) when inoculations were made at three places on one side of the midvein the virus gradually spread to the midvein, crossed it, and spread slowly out along the secondary veins. Except for initial long-distance carriage of virus following inoculation there is no evidence of any type of spread in these leaves other than slow cell-to-cell movement. The fact that the patterns follow the veins probably has nothing to do with veins as conductors of virus, but means only that there is an abundance of parenchyma tissue in which the virus can multiply. The value of a white strain of the virus in a study of virus distribution is evident.

#### COMPARISON OF FIVE VIRUS STRAINS

The study of virus spread in susceptible burley plants was continued by the use of nine plants for inoculations with five distinct strains of the tobacco mosaic virus. One strain was the pure white mosaic used in the previous test, two were collected as white mosaics but were recognized as different strains, one was a yellow mosaic, and one a "burning" strain of a green mosaic. The plants inoculated were grown in a greenhouse ground bench and were as large as vigorously growing field plants. Each was topped at a height of about 5 feet. Plants were inoculated on the tips of the top leaves (plants 1, 3, 5, and 7) or on the edge of one side of lower leaves 6, 7, and 8 about midway between tip and base (plants 2, 4, 6, and 8) or on the top three and lower three leaves (plant 9). Records were kept of the time of invasion of the various suckers, appearance of local lesions, visible spread into veins of inoculated leaves, and spread into the base of uninoculated leaves. The records are given in table 2. Symptoms appeared about as follows: Chlorosis in inoculated areas in about 9 days, with vein clearing in leaves of the top suckers appearing almost simultaneously on top-leaf-inoculated plants, and about 6 days later in bottom-leaf-inoculated plants. Vein clearing in the lower sucker appeared 5 to 11 days after the local lesions, and in all instances but two occurred after the first appearance in the top suckers. This may have been caused in part by the slower growth of the bottom suckers. Visible spread of symptoms along the veins of inoculated leaves required from 16 to 29 days, during which time the chlorotic area at the point of inoculation gradually increased in size. Uninoculated leaves were uninvaded so far as visible symptoms were concerned until 30 days after inoculation, when one leaf showed infection. By the 38th day after inoculation other leaves had developed chlorosis. After that invasion was gradual along the midvein and laterals. The blade tissue was the last to be invaded. The burning strain of the virus on plant 9 caused necrosis around the inoculated areas, and 55 days after inoculation the midveins of all leaves showed chlorosis caused by slow spread of the virus. As the burning strains of the virus are commonly found in the field this observation proves that at least some of the common field strains cause symptoms in old as well as in young leaves.

TABLE 2.—*Time of appearance of symptoms in various parts of tobacco plants inoculated with five strains of the tobacco mosaic virus*

Plant No.	Virus	Leaves inoculated	Number of days required for first appearance of—				
			Local lesions	Vein chlorosis in top suckers	Vein chlorosis in lower suckers	Chlorosis in some veins of the inoculated leaf	Chlorosis of mid-veins of uninoculated leaves
1	White A	Top 3	10	9	16	19	38
2	do	Bottom 6, 7, and 8	9 to 16	16	16		
3	White B	Top 3	9	9	16	19	38
4	do	Bottom 6, 7, and 8	10	16	21	29	43
5	Yellow	Top 3	9	10	16	16	30
6	do	Bottom 6, 7, and 8	9	16	17	24	43
7	Pure white	Top 3	9	9	14	16	38
8	do	Bottom 6, 7, and 8	9	17	14	19	47
9	Burn mosaic	Top 3 and bottom 6, 7, and 8	21	9	16	31	43

Twenty-eight days after inoculation 6 disks 1.5 cm. in diameter were cut from the right side of each uninoculated leaf with a cork borer. The 6 disks were ground together in 0.5 cc. of  $\frac{M}{10}$  disodium phosphate and rubbed on an interveinal area of at least 40 cm.<sup>2</sup> of a burley test plant. From the inoculated leaves 12 disks were cut, 6 from green and 6 from chlorotic areas. The results of the inoculations are given in table 3. They indicate that 28 days after inoculation there had been practically no invasion of uninoculated leaves by any one of the five virus strains used; that invasion of inoculated leaves was not yet complete, with the possible exception of the top leaves inoculated with the burning strain; and that the distribution of the virus is indicated fairly accurately by the development of chlorosis as healthy tissue is invaded. There appears to be no significant difference in virus distribution within the plant between any of the five virus strains tested, which suggests that results obtained with the strain pure white can be considered as about typical of what may be expected from the ordinary field strains so far as plant invasion is concerned.

TABLE 3.—*Approximate number of necrotic spots produced on 40 cm.<sup>2</sup> of leaf of burley test plants rubbed with a composite of six disks per leaf*

[y indicates disks from chlorotic areas, all others from green areas; disks cut 28 days after inoculation]

Leaf No. (numbered from the bottom)	Number of necrotic spots produced on plant No.—								
	1	2	3	4	5	6	7	8	9
4	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0 y-1 0
6	0	0 y-150	0	0 y-30	0 y-2 60	0 y-200	0	0 y 100	0 y 70
7	0	0 y 150	0	0 y-150	0	0 y-100	0	0 y 70	0 y-65
8	0	0 y-80	0	0 y-200	0	0 y-200	0	0 y-80	0
9 to 16	0	0	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0 y-100	0	3
18	0	0	0 y-150	0	0	0	2 y-150	0	3 200 y 200
19	0 y-200	0	0 y-150	0	0	0	0 y-70	0	3 200 y-200
20	50 y-200	0	30 y-200	0	0	0	0	0	3 200 y-200
21	60 y-200			0	0	0		0	
Leaf from 1 top sucker	200	150	200	200	40	200	70	200	200

<sup>1</sup> Leaf old and generally chlorotic and green burning strain which does not cause much chlorosis.

<sup>2</sup> Leaf invaded at base of midrib.

<sup>3</sup> Green areas slightly mottled.

## VIRUS DISTRIBUTION IN FIELD-GROWN PLANTS

A study somewhat similar to the greenhouse studies recorded above was made on field-grown Kentucky No. 16 burley plants. The presence of chlorosis was used to indicate the distribution of the white mosaic virus within the plant. One hundred and twenty White Burley plants were inoculated immediately after topping as follows: 30 plants on the tip of the upper leaf; 30 plants on the tip of a lower green leaf; 30 plants on the broken end of the stalk left when the top was broken out; and 30 plants topped with hands wet with juice from a pure white mosaic plant.

The results at the end of 25 days when the remainder of the crop was harvested can be briefly summarized. Plants inoculated on the tip of the upper leaf had developed local chlorotic lesions around the points of inoculation, and chlorosis had spread a few inches along the midvein and lateral veins or throughout the leaf, depending upon whether the leaf was fully grown or was immature at the time of inoculation. The top suckers developed mosaic. No leaves other than the inoculated leaf and sucker leaves showed any sign of invasion. Only 3 of 30 plants inoculated on a lower leaf developed mosaic. The season was dry and the lower leaves yellowed and died before harvest. Of those inoculated on the broken end of the stalk all but 1 developed mosaic in the upper suckers; 10 less mature plants showed occasional yellow spots up to 2 or 3 inches in diameter and occasional yellow veins on the upper 2 or 3 leaves. The virus had evidently been carried rather quickly into these leaves, as many of the spots were present on them within 10 days after inoculation and increased in size during the next 2 weeks. The remaining leaves on these plants were free from chlorotic spots and both they and the green portions of the invaded leaves presumably were virus-free. All of the plants inoculated by topping with contaminated hands developed mosaic in the upper suckers and 16 plants had scattered chlorotic veins on the upper 2 or 3 leaves similar to those in the group inoculated on the broken end of the stalk. The more advanced the stage of bloom at topping time the less the likelihood of invasion of the upper leaves.

## SUMMARY

From the greenhouse and field studies reported here it appears that highly susceptible burley tobacco is invaded very slowly following inoculation at topping if the leaves have attained almost full size when the plant is inoculated. The top leaves of plants not yet in bloom become invaded by the virus in scattered spots, but the leaves age sufficiently before the virus becomes general so that it is localized to some extent in spots, which slowly increase in size. The advance of virus into mature leaves is extremely slow. At the end of a month uninoculated leaves of inoculated greenhouse plants were virus-free; at the end of 2 months the midveins and laterals of some leaves were invaded, though the blade tissue still remained free from virus. From the practical standpoint the results mean that infection at topping can have little or no influence on quality of the cured leaf of burley tobacco if the plants are topped in the full-bloom stage. If they are topped early, when the flower buds are just beginning to show, some upper leaves may be affected.





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## THE DISTRIBUTION AND RELATION OF FIBER POPULATION, LENGTH, BREAKING LOAD, WEIGHT, DIAMETER, AND PERCENTAGE OF THIN-WALLED FIBERS ON THE COTTONSEED IN FIVE VARIETIES OF AMERICAN UPLAND COTTON<sup>1</sup>

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### INTRODUCTION

The main object of the work presented in this paper was to determine the distribution and relation of fiber population, length, breaking load, weight, diameter, and percentage of thin-walled fibers on the cottonseed in five varieties of American upland cotton (*Gossypium hirsutum* L.).

Cotton fibers are elongated epidermal cells, or hairs, the cellulose walls of which may show a wide variation in thickness on a single seed of a selected variety of cotton. Fibers on a single seed also usually vary considerably in diameter, length, strength, and unit weight. Since a commercial lot of cotton is derived from many seeds coming from many plants, it is apparent that the variation of any physical fiber property in a commercial sample depends upon the variation on single seeds, between seeds, and between plants. Uniformity in any particular fiber property, such as length, is something which the cotton breeder looks for in making plant selections. If there are inherent differences between plants, the breeder can isolate the plants which show relatively high uniformity for the various physical fiber properties and thus develop superior strains. The success which the breeder may have in making plant selections for fiber properties is limited by the degree of variability of these properties found upon the seeds of a plant. Thus it appears that investigators need much information about the location, the population, and the physical properties of fibers on the seed coat.

Literature on this subject is limited. The author<sup>2</sup> found that the fiber population is sparse at the micropylar end of the seed and becomes denser toward the chalazal region, being densest at the base and around the point where the vascular bundle separates into primary veins. According to Campbell,<sup>3</sup> the percentage of undeveloped fibers (having thin walls) is highest at the basal end of the seed,

<sup>1</sup> Received for publication March 29, 1940. Technical Paper No. 117, Department of Agronomy, North Carolina Agricultural Experiment Station. This paper includes a thesis submitted to Duke University in partial fulfillment of the requirements for the degree of doctor of philosophy, awarded June 5, 1939.

<sup>2</sup> MOORE, JERRY H. A STUDY OF THE DISTRIBUTION OF THE FIBER POPULATION ON THE SEED COAT OF NAKED-SEED COTTON AND OTHER TYPES. 1925. [Unpublished master's thesis. Copy on file N. C. State Col. Libr.]

<sup>3</sup> CAMPBELL, ROY C. SOME FACTORS WHICH INFLUENCE THE DEVELOPMENT OF THE COTTON FIBER CELL WALL. 1926. [Unpublished master's thesis. Copy on file N. C. State Col. Libr.]



becoming lower as the pointed end is approached. He states that this fact, together with the previous research on fiber distribution on the seed coat, indicates that the percentage of undeveloped fibers increases as the density of fiber population increases. Campbell concludes that the percentage of undeveloped fibers on a single seed or plant may be influenced by variation in fiber length, the area of longer fibers being associated with the greater percentage of undeveloped fibers. Koshal and Ahmad<sup>4</sup> studied the fibers on single seeds of Surat and Standard Indian cottons. They report that the mean fiber length for fibers at the base of the seed is significantly greater than for fibers at the apex of the seed; that the mean fiber weight per unit of length and the mean fiber strength are significantly greater for the apical than for the basal fibers; that the values of the mean fiber lengths, fiber-length distribution, fiber weight, and fiber strength are practically the same for the right and the left flanks of the seed coat; and that there is a distinct tendency for a high ginning percentage to be associated with low percentage differences between mean fiber length of hairs taken from the apex and the base of a seed.

### MATERIALS AND METHODS

Five improved commercial varieties of American upland cotton bearing a full coat of fuzz hairs on the seed were selected as material for the investigations. Previous measurements on these varieties had indicated that they show differences relative to certain boll, seed, and fiber properties.

The five varieties were grown at Raleigh, N. C., during the 1937 season, with two one-row replications for each variety. The varieties received the same cultural and fertilizer treatment, which was similar to that usually given cotton, and the soil appeared to be uniform. Therefore variation caused by environment was probably not important. The planting plan is given in table 1.

TABLE 1.—*Planting plan of the five cotton varieties grown for the fiber distribution and correlation studies*

Series No.	Variety	Row No. 1	Series No.	Variety	Row No. 1
1	Mexican 128	1	2	Mexican 128	6
	Coker-Cleveland 884-4	2		Coker-Cleveland 884-4	7
	Farm Relief No. 1	3		Farm Relief No. 1	8
	Acala 4067	4		Acala 4067	9
	Rowden 40	5		Rowden 40	10

<sup>1</sup> All rows 150 feet long.

When most of the bolls on the cotton plants had matured and opened, one lock was picked from each of 25 plants of each variety and series. These locks (a lock is the seed cotton in a locule) were picked only from those bolls which appeared to be normal in appearance and free of disease and insect injury. The sample for each variety was put into a paper bag, which was then properly labeled and stored in the laboratory.

In the laboratory 5 locks from each series of each variety were picked at random from the bags containing 25 locks, giving a total sample of 10 locks of each variety. One seed from the middle of each

<sup>4</sup> KOSHAL, RAM SARAN, and AHMAD, NAZIR. VARIATIONS IN THE PROPERTIES OF COTTON FIBRE IN RELATION TO ITS POSITION ON THE SURFACE OF THE SEED. *Textile Inst. Jour.* 23: T211-T296, illus. 1932.

of 10 locks of each variety was carefully removed so as to leave the attached fibers intact. Such a procedure gave a sample of 10 seeds from 10 random plants of each variety.

A leather punch was used to cut out definite areas from the seed coat with the fibers attached. The author<sup>5</sup> had previously used a different method for counting the fiber population. In the earlier investigation the fibers had been pulled from mature seeds taken from bolls preserved in formalin before they opened, the seeds had been dipped for a few seconds in a staining solution, sections had been cut free-hand from six regions on the seed and mounted on a glass slide, and the number of fiber scars (all fiber scars were stained) had been counted by the aid of a microscope. Knowing the area of the microscopic field, the author had then calculated the fiber population per square millimeter of the seed coat.

In the problem presented in this paper, however, it was necessary that all the fibers from each point on the seed coat be counted and also saved for measurement of some of their physical properties. For this reason the leather punch was used in preference to the method just described. A seed was cut transversely into three parts by using a scalpel, and the embryo was removed. Care was observed to avoid cutting or losing fibers from the selected regions on the seed coat. The cutting with the leather punch was done from the inside of the seed coat. When the punch had just about passed through the seed coat, pressure was released and the circular area carefully removed. Six regions, or locations, numbered one to six, were cut from each seed, the area of each region being 3.567 mm.<sup>2</sup> The location of these six regions is shown in figure 1. Each individual punch sample from each seed was placed in a folded, labeled paper, and all the punch samples for the seed were put into a paper bag marked with the variety and plant number.

Since the unit fiber weight, the fiber length, and the fiber strength are affected by variations in temperature and the moisture content of the air, measurement of these fiber properties was made in a laboratory where a constant temperature of 70° F. and a relative humidity of 65 percent were maintained.

The seed coat and fuzz hairs were removed from each punch sample by use of a fine steel comb and the fingers. The remaining tuft of fibers in the punch sample was then weighed on an assay balance to the nearest one-hundredth of a milligram. For region 1, one-tenth of the total weight of the punch sample was used for counting the fiber population and determining the fiber length, unit weight, strength, diameter, and percentage of thin-walled fibers; for the other regions one-fourth of the weight of the punch sample was used for obtaining the fiber data just mentioned.

The fibers were counted on a black velvet pad by using a pair of fine tweezers. Since the fibers were counted in only a fraction of the punch sample, the total population for region 1 was obtained by multiplying the fractional population by 10, and the total population of the other regions was obtained by multiplying the fractional population by 4.

The average length of the fibers on each of the six regions was obtained by measuring the individual lengths of 20 fibers taken at random from a fractional part of a punch sample, summing the

<sup>5</sup> See footnote 2.

individual lengths, and dividing by the number of fibers. Each fiber was straightened out on a black velvet pad and then measured to the nearest sixteenth of an inch by means of a small rule.

The fiber weight per inch of each of the six regions was determined as follows: The total number of fibers in each region was multiplied by the average fiber length of the region, giving the total number of

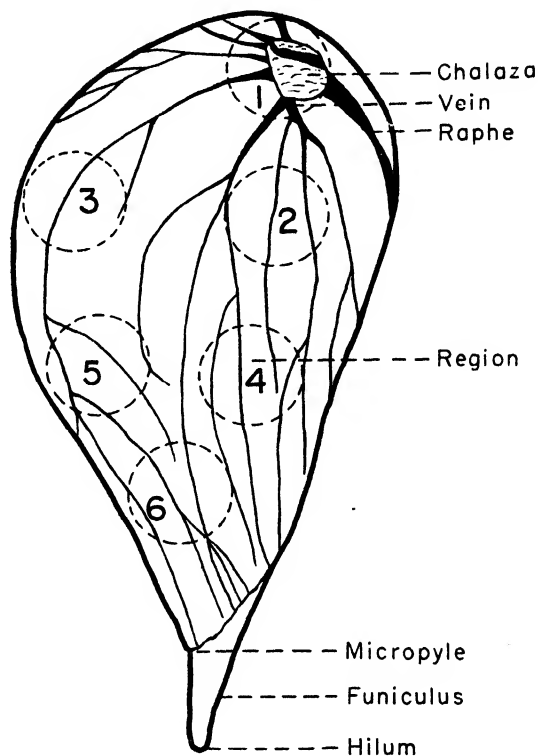


FIGURE 1. Cottonseed showing the location on the seed coat of the regions selected for studying the distribution and relation of the six fiber characters. Each circle with a dotted line represents a region, or punch area, amounting to 3.567 mm.<sup>2</sup> A plane drawn through the long axis of the seed in such a way as to divide the raphe and regions 1, 3, 5, and 6 into two equal parts would cut the seed into halves. The approximate venation of the seed coat is shown. The fuzz hairs (very short fiber) and the lint hairs (long hairs or commercial fibers) are not shown.

inches of fiber in that region; the total number of inches was then divided into the total fiber weight of the region to obtain the average fiber weight per inch.

The breaking load, or strength,<sup>6</sup> of single fibers was measured upon a testing machine of the balance type. A thin strip of rubber was put on each metal jaw of the testing machine in order to prevent the fiber from slipping and also to avoid crushing the fiber by the jaws. Twenty fibers were taken at random from each region and broken individually. The breaking load was added to the testing beam at

<sup>6</sup> Fiber strength and fiber breaking load mean the same thing in this report; the latter terminology is probably the better.

the rate of one-tenth of a gram per second. The breaking load per fiber was recorded to the nearest one-hundredth of a gram. Three-eighths of an inch at the midportion of the fiber length was exposed to the breaking load. The average breaking load per fiber for each region of each variety was obtained by summing that of 20 single fibers and then dividing the total by 20.

The diameter of fibers on each region was determined from mercerized samples. A small bundle of fibers from each region of the 10 seeds of each variety was mercerized thoroughly in an 18-percent solution of sodium hydroxide and then washed in water and air-dried. The fibers were then placed parallel to one another on a glass slide and mounted in liquid paraffin under a cover slip. By the use of a microprojector apparatus the width of each fiber was measured at the midportion of its length. Twenty fibers from each region on the seed coat were measured, and the average diameter of this number was then calculated.

The 20 fibers which had been mounted for diameter measurements were observed under the polarizing microscope in order to estimate the percentage of thin-walled fibers. The percentage of thin-walled fibers was obtained by observing the kind and intensity of colors and the morphological appearance of each fiber.

## EXPERIMENTAL RESULTS

### VARIANCE OF FIBER CHARACTERS WITHIN VARIETIES

The distribution of the fiber population in the Mexican 128 variety is given by plants and regions in table 2. Since each plant is represented by only one seed, allowance must be made for the probability that the data from one seed are not necessarily representative for the whole plant. In table 2 plants, or seeds, are designated by the letters A, B, C, etc., whereas the regions on the seed coat are numbered 1, 2, 3, 4, 5, and 6. Fiber population per square millimeter of any mean in table 2 and all other tables for fiber population can be obtained by dividing the mean by 3.567 (the area of each region). Plant mean as used in this paper is the average of the six regions on the seed.

TABLE 2.—*The analysis of variance of the fiber population by plants and regions on the seed coat in the Mexican 128 variety of cotton*

Plant	Fiber population for region:						Plant totals	Plant means
	1	2	3	4	5	6		
	Number	Number	Number	Number	Number	Number	Number	Number
A	2,310	432	500	181	412	404	4,239	707
B	2,070	240	496	148	336	208	3,438	583
C	1,480	200	240	272	307	492	2,991	499
D	492	208	440	868	276	404	2,728	455
E	1,350	488	278	206	276	358	3,066	511
F	1,600	208	392	180	312	328	3,320	553
G	1,720	300	484	216	352	312	3,384	564
H	680	272	1,312	172	424	124	3,284	547
I	1,110	160	266	464	236	248	2,514	419
J	1,120	392	356	124	356	244	2,572	429
Region total	14,572	2,660	4,764	2,861	3,267	3,172	31,506	
Region means	1,457	296	476	286	327	317		

TABLE 2.—*The analysis of variance of the fiber population by plants and regions on the seed coat in the Mexican 128 variety of cotton—Continued*

## ANALYSIS OF VARIANCE

Variation due to—	Degrees of freedom	Mean square	F			Odds
			Found <sup>1</sup>	Required		
				5 percent point	1 percent point	
Plants	9	43, 643	0.51	2.71	4.31	<19:1
Regions	5	2, 126, 722	24.65	2.42	3.45	>99:1
Error	45	86, 260				
Total	59	Standard deviation of the experiment=204				

## t TEST

Item	Standard error, any mean	Standard error of the difference between any 2 means	Least difference necessary for odds of—	
			19:1	99:1
Plants	120	170	342	457
Regions	93	132	266	355

<sup>1</sup> 43,643/86,260=0.51, and 2,126,722/86,260=24.65.

Analysis of variance<sup>7</sup> for the data is also given in table 2.<sup>8</sup> It is realized that the groups of data in this table and also in other tables of this paper relative to fiber population and percentage of thin-walled fibers, respectively, may not necessarily fit the homogeneous requirements for the application of variance analysis. However, the individual standard errors for columns or items were calculated and tests made for significance. These tests indicated that interpretation of the results through the use of analysis of variance was satisfactory. The groups of data used for obtaining the values for the analysis of variance in the tables relative to the percentage of thin-walled fibers are analogous to weighted data. The word "percentage" is used in order to avoid the term "immaturity." The results indicate no significant differences between mean populations of plants, since the *F* found is only 0.51 and the *F* value required for odds of 19:1 amounts to 2.71. Odds of greater than 19:1 indicate that differences are significant, and odds greater than 99:1 indicate that differences are highly significant. Between the mean populations of the regions the odds are greater than 99:1, indicating high significance, the required *F* value for odds of 99:1 being 3.45, while the mean square of regions divided by the mean square of the remainder amounts to 24.65, indicating that without doubt the odds are greater than 99:1.

On further analysis, the standard error of any region mean is found to be 93, while the standard error of the difference between any two

<sup>7</sup> FISHER, R. A. STATISTICAL METHODS FOR RESEARCH WORKERS. Ed. 5, rev. and enl., 319 pp., illus. Edinburgh and London, 1934.

<sup>8</sup> The *F* and *t* values for this and all other tables in this paper were obtained from SNEDECOR, GEORGE W. CALCULATION AND INTERPRETATION OF ANALYSIS OF VARIANCE AND COVARIANCE. 96 pp. Ames, Iowa. (Iowa State Col., Div. Indus. Sci. Monog. 1.) 1934. See pp. 88-91.

region means is recorded as 132.<sup>9</sup> Then from *t* values and the indicated calculations, least differences amounting to 266 fibers for odds of 19:1 and to 355 fibers for odds of 99:1 are found. The mean of region 1 minus the mean of region 3 equals 1,457 minus 476, or 981 fibers. Since 981 is greater than 355, it appears that region 1 certainly has a denser population than region 3 and therefore a denser population than that of any other region. All possible comparisons of regions 2 to 6, inclusive, indicate no significant difference between the means of any pair of these regions.

Since the odds of less than 19:1 in table 2 do not indicate significant differences between plant means, no comparisons for least differences between plants are given.

The fiber length of the Mexican 128 variety is shown by plants and regions in table 3. The plants, or seeds, and the regions are the same as those in table 2, the only difference being that fiber length instead of population is analyzed. The values for the analysis of variance relative to fiber length give odds of more than 99:1 for plant differences and odds less than 19:1 for region differences. As previously described for table 2, least differences can be used to compare any pair of plants or any pair of regions relative to differences between means.

TABLE 3.—*The analysis of variance of the fiber length by plants and regions on the seed coat in the Mexican 128 variety of cotton*

OBSERVED VALUES								
Plant	Average fiber length for region No.—						Plant totals	Plant means
	1	2	3	4	5	6		
	<i>Inches</i>	<i>Inches</i>	<i>Inches</i>	<i>Inches</i>	<i>Inches</i>	<i>Inches</i>		
A	1.07	0.99	0.98	0.95	1.09	1.05	6.13	1.022
B	1.08	1.14	1.07	.91	.94	1.08	6.22	1.037
C	1.15	1.18	1.16	1.26	1.23	1.20	7.18	1.197
D	1.00	1.11	1.07	.79	1.00	.96	5.93	.988
E	1.01	1.16	1.00	1.24	1.07	.96	6.44	1.073
F	1.20	1.05	1.05	1.16	1.14	1.05	6.65	1.108
G	1.12	1.19	1.18	1.12	1.15	1.13	6.89	1.148
H	1.26	1.24	1.30	1.25	1.11	1.21	7.37	1.228
I	1.38	1.11	1.30	1.32	1.28	1.21	7.60	1.267
J	1.16	1.07	1.05	.98	1.04	1.07	6.37	1.062
Region total	11.43	11.24	11.16	10.98	11.05	10.92	66.78	
Region means	1.143	1.124	1.116	1.098	1.105	1.092		
ANALYSIS OF VARIANCE								
Variation due to—	Degrees of freedom	Mean square	F			Odds		
			Found	Required				
				5 percent point	1 percent point			
Plants	9	0.05274	8.56	2.15	2.94	>99:1		
Regions	5	.00352	.57	4.36	9.02	<19:1		
Error	45	.00616						
Total	59		Standard deviation of experiment=0.0785					

<sup>9</sup> Standard error of any region mean =  $294/\sqrt{10} = 93$ . Standard error of the difference between any two region means =  $93\sqrt{2} = 132$ . The value of *t*, corresponding to 45 degrees of freedom, the number on which the error is based, is 2.014 at the 5 percent point, and 2.690 at the 1 percent point; then for between region means, least difference at 5 percent point for odds of 19:1 =  $2.014 \times 132 = 266$ , and at 1 percent point =  $2.690 \times 132 = 355$  for odds of 99:1.

TABLE 3.—*The analysis of variance of the fiber length by plants and regions on the seed coat in the Mexican 128 variety of cotton—Continued*

## t TEST

Item	Standard error, any mean	Standard error of the difference between any 2 means	Least difference necessary for odds of—	
			19:1	99:1
Plants	0.0320	0.0453	0.0912	0.1219
Regions	.0248	.0351	.0707	.0944

The fiber weight per inch of the Mexican 128 variety is given by plants and regions in table 4. The plants, or seeds, and the regions are identical with those in table 2. The values for the analysis of variance in this table show that the odds for differences in fiber weight are more than 19:1 between plants and greater than 99:1 between regions.

The fiber strength of the Mexican 128 variety is presented in table 5 wherein the values for the analysis of variance indicate real differences in the average fiber breaking load between plants and also between regions.

TABLE 4.—*The analysis of variance of the fiber weight per inch by plants and regions on the seed coat in the Mexican 128 variety of cotton*

## OBSERVED VALUES

Plant	Average fiber weight for region No.						Plant totals	Plant means
	1	2	3	4	5	6		
	0.0001 mg.	0.0001 mg.	0.0001 mg.	0.0001 mg.	0.0001 mg.	0.0001 mg.	0.0001 mg.	0.0001 mg.
A	34	44	46	64	49	55	292	49
B	25	55	40	58	54	54	286	48
C	33	51	38	41	46	44	253	42
D	51	67	45	60	65	60	348	58
E	41	43	63	52	65	61	325	54
F	31	62	49	47	49	51	280	48
G	32	36	41	55	43	52	259	43
H	48	59	39	54	50	57	307	51
I	34	75	39	43	55	57	303	51
J	34	50	50	71	54	66	325	54
Region total	363	542	450	545	530	557	2,987	
Region means	36	54	45	55	53	56		

## ANALYSIS OF VARIANCE

Variation due to—	Degrees of freedom	Mean square	F			Odds
			Found	Required		
				5 percent point	1 percent point	
Plants	9	145.67	2.46	2.15	2.94	>19:1
Regions	5	583.60	9.87	2.42	3.45	>99:1
Error	45	59.13				
Total	59	Standard deviation of the experiment = 7.69				

TABLE 4.—*The analysis of variance of the fiber weight per inch by plants and regions on the seed coat in the Mexican 128 variety of cotton—Continued*

## t TEST

Item	Standard error, any mean	Standard error of the difference between any 2 means	Least difference necessary for odds of—	
			19:1	99:1
Plants	3.14	4.44	8.94	11.94
Regions	2.43	3.44	6.93	9.25

TABLE 5.—*The analysis of variance of the fiber breaking load by plants and regions on the seed coat in the Mexican 128 variety of cotton*

## OBSERVED VALUES

Plant	Average fiber breaking load for region No.—						Plant totals	Plant means
	1	2	3	4	5	6		
	Grams	Grams	Grams	Grams	Grams	Grams	Grams	Grams
A	4.45	4.75	5.15	6.63	7.45	6.95	35.38	5.90
B	3.35	5.70	4.65	5.30	6.08	6.08	32.76	5.46
C	3.43	5.73	4.48	6.43	6.73	5.80	32.60	5.43
D	3.88	6.93	4.95	4.53	7.35	5.13	32.77	5.46
E	4.23	4.88	5.48	7.65	6.80	7.60	36.64	6.11
F	2.78	6.23	4.40	4.40	5.20	4.35	27.36	4.56
G	3.38	4.08	3.78	6.48	4.68	6.08	29.38	4.90
H	5.55	6.65	5.85	6.50	5.70	7.10	37.35	6.23
I	3.58	4.15	3.90	5.73	5.15	5.63	28.14	4.69
J	4.15	5.23	4.98	7.13	5.68	5.70	32.87	5.48
Region total	38.78	55.33	47.62	60.78	61.42	61.32	325.25	
Region means	3.88	5.53	4.76	6.08	6.14	6.13		

## ANALYSIS OF VARIANCE

Variation due to	Degrees of freedom	Mean square	F			Odds
			Found	Required		
				5 percent point	1 percent point	
Plants	9	1.9333	2.88	2.15	2.94	>19:1
Regions	5	8.5693	12.77	2.42	3.45	>99:1
Error	45	.6711				
Total	59	Standard deviation of experiment = 0.819				

## t TEST

Item	Standard error, any mean	Standard error of the difference between any 2 means	Least difference necessary for odds of	
			19:1	99:1
Plants	0.334	0.472	0.951	1.270
Regions	.250	.366	.737	.985



The variance analysis for the percentage of thin-walled fibers in Mexican 128, given in table 6, indicates highly significant differences between regions and no differences between plants.

TABLE 6.—*The analysis of variance of the percentage of thin-walled fibers by plants and regions on the seed coat of the Mexican 128 variety of cotton*

## OBSERVED VALUES

Plant	Thin-walled fibers for region No.—						Plant totals	Plant means
	1	2	3	4	5	6		
	Percent	Percent	Percent	Percent	Percent	Percent	Percent	Percent
A.....	18	5	20	5	10	17	75	13
B.....	50	5	20	0	0	5	80	13
C.....	33	5	20	0	5	0	63	11
D.....	20	10	9	47	5	5	96	16
E.....	45	14	0	16	5	5	85	14
F.....	42	15	27	0	5	0	89	15
G.....	55	42	35	5	14	0	151	25
H.....	32	16	60	0	5	10	123	21
I.....	31	10	24	5	0	5	77	13
J.....	35	29	20	0	15	5	104	17
Region total .....	363	151	235	78	64	52	943	.....
Region means.....	36	15	24	8	6	5	.....	.....

## ANALYSIS OF VARIANCE

Variation due to—	Degrees of freedom	Mean square	F			Odds
			Found	Required		
				5 percent point	1 percent point	
Plants.....	9	113.00	0.81	2.71	4.31	<19:1
Regions.....	5	1,489.40	10.73	2.42	3.45	>99:1
Error.....	45	138.84				
Total.....	59	Standard deviation of the experiment=11.8				

## t TEST

Item	Standard error, any mean	Standard error of the difference between any 2 means	Least difference necessary for odds of—	
			19:1	99:1
Plants.....	4.816	6.811	13.72	18.32
Regions.....	3.732	5.278	10.63	14.20

The analysis of variance of the fiber diameter by plants and regions in Mexican 128 is given in table 7. The values show that there are highly significant differences between plants and also between regions.

TABLE 7.—The analysis of variance of the fiber diameter by plants and regions on the seed coat of the Mexican 128 variety of cotton

OBSERVED VALUES								
Plant	Average fiber diameter for region No.—						Plant totals	Plant means
	1	2	3	4	5	6		
	<i>Microns</i>	<i>Microns</i>	<i>Microns</i>	<i>Microns</i>	<i>Microns</i>	<i>Microns</i>	<i>Microns</i>	<i>Microns</i>
A.....	16.49	17.80	17.54	16.75	17.54	17.54	103.7	17.28
B.....	15.45	15.96	15.71	14.13	14.40	14.40	90.1	15.02
C.....	16.23	15.96	16.49	14.92	14.66	14.92	93.2	15.53
D.....	18.33	17.28	16.49	16.49	17.28	17.80	103.7	17.28
E.....	16.49	18.33	17.54	17.02	17.28	18.06	104.7	17.45
F.....	16.49	17.54	17.05	15.71	15.45	14.66	96.9	16.15
G.....	15.96	15.71	16.23	16.49	15.18	16.49	96.1	16.02
H.....	16.75	16.23	14.66	15.96	13.35	16.75	93.7	15.62
I.....	14.40	18.33	17.02	14.66	15.71	17.02	97.1	16.18
J.....	16.49	17.02	16.75	17.54	15.71	16.49	100.0	16.67
Region total	163.1	170.2	165.5	159.7	156.6	164.1	970.2	
Region means	16.31	17.02	16.55	15.97	15.66	16.41		

ANALYSIS OF VARIANCE						
Variation due to—	Degrees of freedom	Mean square	F			Odds
			Found	Required		
				5 percent point	1 percent point	
Plants .....	9	4.14	7.11	2.15	2.94	>99:1
Regions .....	5	2.22	3.81	2.42	3.45	>99:1
Error .....	45	.582				
Total .....	59		Standard deviation of the experiment = 0.762			

## t TEST

Item	Standard error, any mean	Standard error of the difference between any 2 means	Least difference necessary for odds of—	
			19:1	99:1
Plants.....	0.3110	0.4398	0.89	1.18
Regions.....	.2410	.3408	.69	.92

The data for the Mexican 128 variety have been presented in detailed form in tables 2, 3, 4, 5, 6, and 7 in order to give an example of the procedure used in recording the measurements of fiber population, length, weight, breaking load, percentage of thin-walled fibers, and diameter. For other varieties and for totals of all varieties, only the means and the values for the analysis of variance are given.

The values for the analysis of variance in the fiber population of the Coker-Cleveland 884-4 variety are given in table 8, wherein odds of less than 19:1 indicate no significant differences between plant means whereas odds greater than 99:1 show that differences between region means are highly significant.

TABLE 8.—*The analysis of variance of the fiber population by plants and regions on the seed coat in the Coker-Cleveland 884-4 variety of cotton*

REGION MEANS										
Region No	1	2	3	4	5	6				
Mean number of fibers	2,044	437	513	273	334	241				
PLANT MEANS										
Plant symbol	A	B	C	D	E	F	G	H	I	J
Mean number of fibers	783	649	695	871	380	692	561	599	608	563
ANALYSIS OF VARIANCE										
Variation due to	Degrees of freedom	Mean square	F			Odds				
			Found	Required						
				5 percent point	1 percent point					
Plants	9	108,054	1.30	2.15	2.94	< 19:1				
Regions	5	4,830,758	57.95	2.42	3.45	> 99:1				
Error	45	83,306								
Total	59	Standard deviation of the experiment = 289								

## t TEST

Item	Standard error, any mean	Standard error of the difference between any 2 means	Least difference necessary for odds of—	
			19:1	99:1
Plants	118	167	336	449
Regions	91	129	200	347

<sup>1</sup> Probably not valid for certain comparisons. Least differences calculated from individual standard errors of regions are as follows for these comparisons:

Regions compared	Least difference for odds of	
	19:1	99:1
2 and 4	121	166
2 and 6	106	145
3 and 4	143	196
3 and 5	145	198
3 and 6	132	180
5 and 6	81	111

The values for the fiber length in the Coker-Cleveland 884-4 variety are given in table 9, wherein odds greater than 19:1 indicate significant differences between plant means as well as region means.

TABLE 9.—*The analysis of variance of the fiber length by plants and regions on the seed coat of the Coker-Cleveland 884-4 variety of cotton*

REGION MEANS										
Region No	1	2	3	4	5	6				
Mean fiber length (inches)	1.112	1.148	1.119	1.127	1.073	1.034				
PLANT MEANS										
Plant symbol	A	B	C	D	E	F	G	H	I	J
Mean fiber length (inches)	1.023	1.110	1.137	1.185	1.095	1.047	1.143	1.142	1.115	1.025
ANALYSIS OF VARIANCE										
Variation due to	Degrees of freedom	Mean square	F			Odds				
			Found	Required						
			5 percent point	1 percent point						
Plants	9	0.0179	2.54	2.15	2.94	>19:1				
Regions	5	.0172	2.46	2.42	3.45	>19:1				
Error	45	.0070								
Total	59	Standard deviation of the experiment = 0.0840								

## t TEST

Item	Standard error, any mean	Standard error of the difference between any 2 means	Least difference necessary for odds of	
			19:1	99:1
Plants	0.0343	0.0485	0.098	0.1305
Regions	.0266	.0376	.076	.1011

The variance in the fiber weight of the Coker-Cleveland 884-4 variety is shown in table 10, wherein odds of greater than 99:1 indicate that between plants and also between regions the differences are highly significant.

TABLE 10.—*The analysis of variance of the fiber weight per inch by plants and regions on the seed coat of the Coker-Cleveland 884-4 variety of cotton*

REGION MEANS										
Region No	1	2	3	4	5	6				
Mean fiber weight (0.0001 mg.)	30	48	41	53	52	59				
PLANT MEANS										
Plant symbol	A	B	C	D	E	F	G	H	I	J
Mean fiber weight (0.0001 mg.)	52	40	44	38	56	45	41	47	57	52

TABLE 10.—*The analysis of variance of the fiber weight per inch by plants and regions on the seed coat of the Coker-Cleveland 884-4 variety of cotton—Continued*

Variation due to—	Degrees of freedom	Mean square	F			Odds
			Found	Required		
				5 percent point	1 percent point	
Plants .....	9	264.67	6.91	2.15	2.94	>99:1
Regions .....	5	1,046.20	27.31	2.42	3.45	>99:1
Error .....	45	38.31				
Total .....	59	Standard deviation of the experiment=6.19				

## t TEST

Item	Standard error, any mean	Standard error of the difference between any 2 means	Least difference necessary for odds of—	
			19:1	99:1
Plants.....	2.53	3.58	7.21	9.63
Regions.....	1.96	2.77	5.58	7.45

The variance analysis for the fiber breaking load of the Coker-Cleveland 884-4 variety is presented in table 11, in which the odds are less than 19:1 between plants and more than 99:1 between regions.

TABLE 11.—*The analysis of variance of the fiber breaking load by plants and regions on the seed coat in the Coker-Cleveland 884-4 variety of cotton*

## REGION MEANS

Region No .....	1	2	3	4	5	6
Mean breaking load (grams) .....	2.81	4.47	3.86	5.72	5.21	5.16

## PLANT MEANS

Plant symbol .....	A	B	C	D	E	F	G	H	I	J
Mean breaking load (grams) .....	4.69	4.37	4.61	4.59	4.91	4.09	4.12	4.36	5.13	4.55

## ANALYSIS OF VARIANCE

Variation due to—	Degrees of freedom	Mean square	F			Odds
			Found	Required		
				5 percent point	1 percent point	
Plants .....	9	0.6357	1.57	2.15	2.94	<19:1
Regions .....	5	11.3737	24.15	2.42	3.45	>99:1
Error .....	45	.4040				
Total .....	59	Standard deviation of the experiment = 0.636				

TABLE 11.—*The analysis of variance of the fiber breaking load by plants and regions on the seed coat in the Coker-Cleveland 884-4 variety of cotton—Continued**t* TEST

Item	Standard error, any mean	Standard error of the difference between any 2 means	Least difference necessary for odds of—	
			19:1	99:1
Plants	0.2596	0.3671	0.740	0.988
Regions	.2011	.2844	.573	.765

The data for the analysis of variance of the percentage of thin-walled fibers in the Coker-Cleveland 884-4 variety shown in table 12 indicate that there are highly significant differences between plant means and also between region means.

TABLE 12.—*The analysis of variance of the percentage of thin-walled fibers by plants and regions on the seed coat of the Coker-Cleveland 884-4 variety of cotton*

## REGION MEANS

Region No	1	2	3	4	5	6
Mean percentage of thin-walled fibers	34	14	26	11	12	10

## PLANT MEANS

Plant symbol	A	B	C	D	E	F	G	H	I	J
Mean percentage of thin-walled fibers	19	27	17	17	15	12	21	23	12	13

## ANALYSIS OF VARIANCE

Variation due to	Degrees of freedom	Mean square	F			Odds
			Found	Required		
				5 percent point	1 percent point	
Plants	9	158.67	3.28	2.15	2.94	>99:1
Regions	5	981.80	20.28	2.42	3.45	>99:1
Error	45	48.42				
Total	59	Standard deviation of the experiment = 6.96				

*t* TEST

Item	Standard error, any mean	Standard error of the difference between any 2 means	Least difference necessary for odds of—	
			19:1	99:1
Plants	2.841	1.018	8.09	10.81
Regions	2.201	3.113	6.27	8.37

The values for variance in the fiber diameter of the Coker-Cleveland 884-4 variety presented in table 13 do not indicate significant differences between plants or regions.

TABLE 13.—*The analysis of variance of the fiber diameter by plants and regions on the seed coat of the Coker-Cleveland 884-4 variety of cotton*

REGION MEANS						
Region No.	1	2	3	4	5	6
Mean fiber diameter (microns)	15.66	15.18	15.89	15.81	15.68	15.50

PLANT MEANS										
Plant symbol	A	B	C	D	E	F	G	H	I	J
Mean fiber diameter (microns)	15.36	16.01	15.14	15.31	16.23	15.58	15.36	14.83	16.11	15.97

#### ANALYSIS OF VARIANCE

Variation due to	Degrees of freedom	Mean square	F			Odds
			Found	Required		
				5 percent point	1 percent point	
Plants	9	1.567	2.08	2.15	2.94	19:1
Regions	5	.640	.85	4.36	9.02	19:1
Error	15	.753				
Total	59					

Standard deviation of the experiment = 0.87

Standard deviation of the experiment = 0.87

#### t TEST

Item	Standard error, any mean	Standard error of the difference between any 2 means	Least difference necessary for odds of—	
			19:1	99:1
Plants	0.355	0.502	1.01	1.35
Regions	.275	.389	.78	1.05

The analysis of variance for the fiber population in the Farm Relief No. 1 variety is given in table 14. The odds of less than 19:1 in this table indicate no significant differences between plants, while odds greater than 99:1 point to highly significant differences between regions.

TABLE 14.—*The analysis of variance of the fiber population by plants and regions on the seed coat of the Farm Relief No. 1 variety of cotton*

REGION MEANS						
Region No.	1	2	3	4	5	6
Mean number of fibers	1,493	440	261	233	261	256

TABLE 14.—*The analysis of variance of the fiber population by plants and regions on the seed coat of the Farm Relief No. 1 variety of cotton—Continued*

PLANT MEANS										
Plant symbol.	A	B	C	D	E	F	G	H	I	J
Mean number of fibers	493	551	497	452	439	506	550	476	593	517

ANALYSIS OF VARIANCE						
Variation due to--	Degrees of freedom	Mean square	F			Odds
			Found	Required		
				5 percent point	1 percent point	
Plants	9	13.389	0.84	2.71	4.31	<19:1
Regions	5	2,392.653	149.58	2.42	3.45	>99:1
Error	45	15.996				
Total	59	Standard deviation of the experiment = 126				

t TEST				
Item	Standard error, any mean	Standard error of the difference between any 2 means	Least difference necessary for odds of—	
			19:1	99:1
Plants	51	73	147	196
Regions	49	57	115	153

<sup>1</sup> Probably not valid for certain comparisons. Least differences calculated from individual standard errors of regions are as follows for these comparisons:

Regions compared	Least differences for odds of—	
	19:1	99:1
3 and 4	80	109
3 and 5	86	118
3 and 6	85	117

The variance of the fiber length in the Farm Relief No. 1 variety is presented in table 15, wherein odds of greater than 99:1 show that there are highly significant differences between plants and odds greater than 19:1 indicate that there are probably significant differences in the mean fiber length of regions.

TABLE 15.—*The analysis of variance of the fiber length by plants and regions on the seed coat of the Farm Relief No. 1 variety of cotton*

REGION MEANS						
Region No	1	2	3	4	5	6
Mean fiber length (inches)	1.130	1.181	1.190	1.197	1.153	1.140



TABLE 15.—*The analysis of variance of the fiber length by plants and regions on the seed coat of the Farm Relief No. 1 variety of cotton—Continued*

PLANT MEANS										
Plant symbol.....	A	B	C	D	E	F	G	H	I	J
Mean fiber length (inches)...	1.170	1.180	1.120	1.160	1.120	1.050	1.290	1.230	1.140	1.200

ANALYSIS OF VARIANCE						
Variation due to—	Degrees of freedom	Mean square	F			Odds
			Found	Required		
				5 percent point	1 percent point	
Plants.....	9	0.0265	8.55	2.15	2.94	>99:1
Regions.....	5	.0078	2.52	2.42	3.45	>19:1
Error.....	45	.0031				
Total.....	59	Standard deviation of the experiment=0.0557				

t TEST				
Item	Standard error, any mean	Standard error of the difference between any 2 means	Least difference necessary for odds of—	
			19:1	99:1
Plants.....	0.0227	0.0321	0.0646	0.0863
Regions.....	.0176	.0249	.0501	.0670

The analysis of variance of the fiber weight in the Farm Relief No. 1 variety is given in table 16. The odds of greater than 99:1 therein shown indicate highly significant differences between plants and also between regions.

TABLE 16.—*The analysis of variance of the fiber weight per inch by plants and regions on the seed coat of the Farm Relief No. 1 variety of cotton*

REGION MEANS						
Region No.....	1	2	3	4	5	6
Mean fiber weight (0.0001 mg.).....	34	52	48	59	56	57

PLANT MEANS										
Plant symbol.....	A	B	C	D	E	F	G	H	I	J
Mean fiber weight (0.0001 mg.).....	54	40	49	61	52	65	44	47	45	53

TABLE 16.—*The analysis of variance of the fiber weight per inch by plants and regions on the seed coat of the Farm Relief No. 1 variety of cotton—Continued*

## ANALYSIS OF VARIANCE

Variation due to—	Degrees of freedom	Mean square	F			Odds
			Found	Required		
				5 percent point	1 percent point	
Plants.....	9	366.33	8.77	2.15	2.94	>99:1
Regions.....	5	866.60	20.75	2.42	3.45	>99:1
Error.....	45	41.76				
Total.....	59	Standard deviation of the experiment = 6.46				

## t TEST

Item	Standard error, any mean	Standard error of the difference between any 2 means	Least difference necessary for odds of—	
			19:1	99:1
Plants .....	2.64	3.73	7.51	10.03
Regions .....	2.04	2.88	5.80	7.75

The data for variance of fiber breaking load in the Farm Relief No. 1 variety presented in table 17 indicate nonsignificant differences between plants and highly significant differences between regions.

TABLE 17.—*The analysis of variance of the fiber breaking load by plants and regions on the seed coat of the Farm Relief No. 1 variety of cotton*

## REGION MEANS

Region No. ....	1	2	3	4	5	6
Mean breaking load (grams) .....	3.04	4.52	4.20	5.78	5.45	5.49

## PLANT MEANS

Plant symbol .....	A	B	C	D	E	F	G	H	I	J
Mean breaking load (grams) ..	4.35	4.81	4.96	4.25	4.04	4.90	4.74	4.58	5.50	4.73

## ANALYSIS OF VARIANCE

Variation due to—	Degrees of freedom	Mean square	F			Odds
			Found	Required		
				5 percent point	1 percent point	
Plants.....	9	0.7260	1.40	2.15	2.94	<19:1
Regions.....	5	10.7310	20.63	2.42	3.45	>99:1
Error.....	45	.5202				
Total.....	59	Standard deviation of the experiment=0.721				

TABLE 17.—*The analysis of variance of the fiber breaking load by plants and regions on the seed coat of the Farm Relief No. 1 variety of cotton—Continued*

## t TEST

Item	Standard error, any mean	Standard error of the difference between any 2 means	Least difference necessary for odds of—	
			19:1	99:1
Plants	0.294	0.416	0.838	1.119
Regions	.228	.322	.649	.866

Values for the analysis of variance in the percentage of thin-walled fibers in the Farm Relief No. 1 variety are presented in table 18. Odds greater than 99:1 point to the fact that there are highly significant differences between plant and also region means in this character.

TABLE 18.—*The analysis of variance of the percentage of thin-walled fibers by plants and regions on the seed coat of the Farm Relief No. 1 variety of cotton*

## REGION MEANS

Region No.	1	2	3	4	5	6
Mean percentage of thin-walled fibers	59	11	24	6	7	12

## PLANT MEANS

Plant symbol.	A	B	C	D	E	F	G	H	I	J
Mean percentage of thin-walled fibers	13	32	7	14	13	13	36	29	30	12

## ANALYSIS OF VARIANCE

Variation due to—	Degrees of freedom	Mean square	Found	Required		Odds
				5 percent point	1 percent point	
Plants	9	677.11	4.48	2.15	2.94	>99:1
Regions	5	4,166.20	27.59	2.42	3.45	>99:1
Error	46	151.02				
Total	59	Standard deviation of the experiment = 12.29				

## t TEST

Item	Standard error any mean	Standard error of the differences between any 2 means	Least difference necessary for odds of—	
			19:1	99:1
Plants	5.02	7.10	14.30	19.10
Regions	3.89	5.50	11.08	14.80

The variance analysis for fiber diameter of the Farm Relief No. 1 variety in table 19 indicates highly significant differences between plants and nonsignificant differences between regions.

TABLE 19.—*The analysis of variance of the fiber diameter by plants and regions on the seed coat in the Farm Relief No. 1 variety of cotton*

REGION MEANS										
Region No	1	2	3	4	5	6				
Mean fiber diameter (microns)	17.25	17.04	17.01	16.34	16.70	16.75				
PLANT MEANS										
Plant symbol	A	B	C	D	E	F	G	H	I	J
Mean fiber diameter (microns)	17.19	16.80	15.81	16.76	16.54	17.89	16.93	17.72	16.75	16.14
ANALYSIS OF VARIANCE										
Variation due to—	Degrees of freedom	Mean square	F			Odds				
			Found	Required						
				5 percent point	1 percent point					
Plants	9	2.389	4.68	2.15	2.04	>99:1				
Regions	5	1.090	2.08	2.42	3.45	<19:1				
Error	45	.51								
Total	59	Standard deviation of the experiment = 0.714								

#### t TEST

Item	Standard error, any mean	Standard error of the difference between any 2 means	Least difference necessary for odds of—	
			19:1	99:1
Plants	0.291	0.412	0.83	1.11
Regions	.226	.320	.64	.86

The variance analysis for the distribution of the fiber population on the seed coat of the Acala 4067 variety is presented in table 20, wherein odds less than 19:1 indicate that differences between plants are not significant and odds greater than 99:1 show that there are highly significant differences between regions.

TABLE 20.—*The analysis of variance of the fiber population by plants and regions on the seed of the Acala 4067 variety of cotton*

REGION MEANS						
Region No	1	2	3	4	5	6
Mean number of fibers	1,646	499	753	270	395	406

TABLE 20.—*The analysis of variance of the fiber population by plants and regions on the seed of the Acala 4067 variety of cotton—Continued*

PLANT MEANS										
Plant symbol.....	A	B	C	D	E	F	G	H	I	J
Mean number of fibers.....	724	585	433	569	1,035	790	567	701	504	707

Variation due to—	Degrees of freedom	Mean square	F			Odds
			Found	Required		
				5 percent point	1 percent point	
Plants .....	9	175, 836	1. 91	2. 15	2. 94	<19:1
Regions .....	5	2, 586, 907	28. 06	2. 42	3. 45	>99:1
Error .....	45	92, 187				
Total .....	59	Standard deviation of the experiment = 304				

t TEST				
Item	Standard error, any mean	Standard error of the difference between any 2 means	Least difference necessary for odds of—	
			19:1	99:1
Plants.....	124	175	352	471
Regions.....	96	136	274	396

<sup>1</sup> Probably not valid for certain comparisons. Least differences calculated from individual standard errors of regions are as follows for these comparisons:

Regions compared	Least difference for odds of—	
	19:1	99:1
2 and 4.....	122	167
3 and 4.....	387	530
3 and 5.....	387	530
3 and 6.....	384	527
4 and 5.....	100	136
4 and 6.....	86	118

The variance analysis for the distribution of the fiber length on the seed coat of the Acala 4067 variety is presented in table 21, wherein odds of greater than 99:1 indicate that there are highly significant differences between region means and also between plant means.

TABLE 21.—*The analysis of variance of the fiber length by plants and regions on the seed coat of the Acala 4067 variety of cotton*

REGION MEANS						
Region No. ....	1	2	3	4	5	6
Mean fiber length (inches) .....	1.030	1.016	1.001	0.982	0.943	0.942

PLANT MEANS										
Plant symbol. ....	A	B	C	D	E	F	G	H	I	J
Mean fiber length (inches) ..	1.000	1.050	1.050	0.960	0.990	1.000	0.960	0.830	0.960	1.060

Variance due to—	Degrees of freedom	Mean square	F			Odds
			Found	Required		
				5 percent point	1 percent point	
Plants .....	9	0.0268	9.93	2.15	2.94	>99:1
Regions .....	5	.0137	5.07	2.42	3.45	>99:1
Error .....	45	.0027				
Total .....	59	Standard deviation of the experiment = 0.052				

## t TEST

Item	Standard error, any mean	Standard error of the difference between any 2 means	Least difference necessary for odds of—	
			19:1	99:1
Plants .....	0.0212	0.0300	0.0604	0.0807
Regions .....	.0164	.0232	.0467	.0624

The analysis of variance for the fiber weight in the Acala 4067 variety is presented in table 22. Odds greater than 99:1 point to highly significant differences between both regions and plants.

TABLE 22.—*The analysis of variance for the fiber weight per inch by plant and regions on the seed coat of the Acala 4067 variety of cotton*

REGION MEANS						
Region No. ....	1	2	3	4	5	6
Mean fiber weight (0.0001 mg.) ..	33	43	41	58	55	58

PLANT MEANS										
Plant symbol. ....	A	B	C	D	E	F	G	H	I	J
Mean fiber weight (0.0001 mg.) ..	40	48	48	49	42	46	47	51	65	46

TABLE 22.—*The analysis of variance for the fiber weight per inch by plant and regions on the seed coat of the Acala 4067 variety of cotton—Continued*

## ANALYSIS OF VARIANCE

Variance due to—	Degrees of freedom	Mean square	F			Odds
			Found	Required		
				5 percent point	1 percent point	
Plants.....	9	260.33	8.72	2.15	2.94	>99:1
Regions.....	5	1,107.40	37.07	2.42	3.45	>99:1
Error.....	45	29.87				
Total.....	59	Standard deviation of the experiment = 5.465				

## t TEST

Item	Standard error, any mean	Standard error of the difference between any 2 means	Least difference necessary for odds of—	
			19:1	99:1
Plants.....	2.23	3.15	6.34	8.47
Regions.....	1.73	2.45	4.93	6.59

The analysis of variance for the fiber breaking load in the Acala 4067 variety is presented in table 23. The values show that there are highly significant differences between plants and also between regions in the fiber strength.

TABLE 23.—*The analysis of variance of the fiber breaking load by plants and regions on the seed coat of the Acala 4067 variety of cotton*

## REGION MEANS

Region No.....	1	2	3	4	5	6
Mean breaking load (grams).....	3.16	4.23	4.22	6.28	5.59	6.01

## PLANT MEANS

Plant symbol.....	A	B	C	D	E	F	G	H	I	J
Mean breaking load (grams).....	3.70	4.60	4.37	5.22	4.16	5.91	5.61	4.75	5.46	5.29

## ANALYSIS OF VARIANCE

Variation due to—	Degrees of freedom	Mean square	F			Odds
			Found	Required		
				5 percent point	1 percent point	
Plants .....	9	2.8487	4.53	2.15	2.94	>99:1
Regions .....	5	15.0388	23.90	2.42	3.45	>99:1
Error .....	45	.6293				
Total .....	59	Standard deviation of the experiment=0.793				

TABLE 23.—*The analysis of variance of the fiber breaking load by plants and regions on the seed coat of the Acala 4067 variety of cotton—Continued**t* TEST

Item	Standard error, any mean	Standard error of the difference between any 2 means	Least difference necessary for odds of—	
			19:1	99:1
Plants .....	0.3237	0.4578	0.922	1.231
Regions .....	.2508	.3547	.714	.954

The variance analysis of the percentage of thin-walled fibers in the Acala 4067 variety is contained in table 24 wherein the values indicate real differences between regions and nonsignificant differences between plants for the percentage means.

TABLE 24.—*The analysis of variance of the percentage of thin-walled fibers by plants and regions on the seed coat of the Acala 4067 variety of cotton*

## REGION MEANS

Region No	1	2	3	4	5	6
Mean percentage of thin-walled fibers .....	59	24	34	4	9	6

## PLANT MEANS

Plant symbol	A	B	C	D	E	F	G	H	I	J
Mean percentage of thin-walled fibers .....	30	18	34	11	32	25	16	23	19	18

## ANALYSIS OF VARIANCE

Variation due to—	Degrees of freedom	Mean square	F		Odds
			Found	Required	
				5 percent point 1 percent point	
Plants .....	9	344.00	1.91	2.15	2.04
Regions .....	4	4,510.40	25.07	2.42	3.45
Error .....	45	179.89			
Total .....	59	Standard deviation of the experiment=13.40			

*t* TEST

Item	Standard error, any mean	Standard error of the difference between any 2 means	Least difference necessary for odds of—	
			19:1	99:1
Plants .....	5.47	7.74	15.59	20.82
Regions .....	4.24	5.99	12.06	16.11



The variance values for the Acala 4067 variety presented in table 25 indicate highly significant differences in fiber diameter between plants and significant differences between regions.

TABLE 25.—*The analysis of variance of the fiber diameter by plants and regions on the seed coat of the Acala 4067 variety of cotton*

REGION MEANS										
Region No. ....	1	2	3	4	5	6				
Mean fiber diameter (microns) .....	15.81	15.16	14.55	15.10	15.10	14.82				
PLANT MEANS										
Plant symbol. ....	A	B	C	D	E	F	G	H	I	J
Mean fiber diameter (microns) .....	14.49	15.23	14.96	15.97	15.27	14.79	14.44	14.26	16.62	14.88
ANALYSIS OF VARIANCE										
Variation due to—	Degrees of freedom	Mean square	F			Odds				
			Found	Required						
				5 percent point	1 percent point					
Plants .....	9	3.200	6.15	2.15	2.94	>99:1				
Regions .....	5	1.760	3.38	2.42	3.45	>99:1				
Error .....	45	.520								
Total .....	59	Standard deviation of the experiment=0.721								

#### t TEST

Item	Standard error, any mean	Standard error of the difference between any 2 means	Least difference necessary for odds of—	
			19:1	99:1
Plants .....	0.294	0.416	0.84	1.12
Regions .....	.228	.322	.65	.87

The variance of the fiber population in the Rowden 40 variety is contained in Table 26. Odds of less than 19:1 indicate that differences between plants are not significant, whereas odds greater than 99:1 show that there are highly significant differences between regions.

TABLE 26.—*The analysis of variance of the fiber population by plants and regions on the seed coat of the Rowden 40 variety of cotton*

REGION MEANS						
Region No. ....	1	2	3	4	5	6
Mean number of fibers .....	1,725	414	424	202	438	216

TABLE 26.—*The analysis of variance of the fiber population by plants and regions on the seed coat of the Rowden 40 variety of cotton—Continued*

PLANT MEANS										
Plant symbol.....	A	B	C	D	E	F	G	H	I	J
Mean number of fibers.....	466	564	627	579	472	719	440	599	750	481

ANALYSIS OF VARIANCE						
Variation due to—	Degrees of freedom	Mean square	F			Odds
			Found	Required		
				5 percent point	1 percent point	
Plants.....	9	60, 446	.64	2.71	4.31	<19:1
Regions.....	5	3, 315, 923	30.59	2.42	3.45	>99:1
Error.....	45	108, 415				
Total.....	59	Standard deviation of the experiment=329				

t TEST				
Item	Standard error, any mean	Standard error of the difference between any 2 means	Least difference necessary for odds of—	
			19:1	99:1
Plants.....	134	190	383	511
Regions.....	104	147	1 296	1 395

<sup>1</sup> Probably not valid for certain comparisons. Least differences calculated from individual standard errors of regions are as follows for these comparisons:

Regions compared	Least differences for odds of—	
	19:1	99:1
2 and 4.....	116	158
2 and 6.....	127	174
3 and 4.....	124	169
3 and 6.....	134	184

The analysis of variance for the fiber length in the Rowden 40 variety is presented in table 27, wherein odds greater than 99:1 indicate that there are highly significant differences between both regions and plants.

TABLE 27.—*The analysis of variance of the fiber length by plants and regions on the seed coat of the Rowden 40 variety of cotton*

REGION MEANS						
Region No.....	1	2	3	4	5	6
Mean fiber length (inches).....	1.053	1.015	1.000	0.992	0.947	0.937

TABLE 27.—*The analysis of variance of the fiber length by plants and regions on the seed coat of the Rowden 40 variety of cotton—Continued*

PLANT MEANS										
Plant symbol.....	A	B	C	D	E	F	G	H	I	J
Mean fiber length (inches) ..	1.090	0.950	1.060	1.010	1.020	0.860	0.910	1.060	0.860	1.060

Variation due to—	Degrees of freedom	Mean square	F			Odds
			Found	Required		
				5 percent point	1 percent point	
Plants..	9	0.0421	14.52	2.15	2.94	>99:1
Regions.....	5	.0187	6.45	2.42	3.45	>99:1
Error.....	45	.0029				
Total.....	59	Standard deviation of the experiment = 0.054.				

t TEST				
Item	Standard error, any mean	Standard error of the difference between any 2 means	Least difference necessary for odds of—	
			19:1	99:1
Plants.....	0.0220	0.0311	0.0626	0.0837
Regions.....	.0171	.0242	.0487	.0651

The analysis of variance for the fiber weight in the Rowden 40 variety is contained in table 28. Odds greater than 19:1 for plants and more than 99:1 for regions show that there are significant differences between both regions and plants.

TABLE 28.—*The analysis of variance of the fiber weight per inch by plants and regions on the seed coat of the Rowden 40 variety of cotton*

REGION MEANS						
Region No. ....	1	2	3	4	5	6
Mean fiber weight (0.0001 mg.).....	38	48	45	65	57	65

PLANT MEANS										
Plant symbol.....	A	B	C	D	E	F	G	H	I	J
Mean fiber weight (0.0001 mg.).....	35	41	57	51	56	53	55	73	60	48

TABLE 28.—*The analysis of variance of the fiber weight per inch by plants and regions on the seed coat of the Rowden 40 variety of cotton—Continued*

## ANALYSIS OF VARIANCE

Variation due to—	Degrees of freedom	Mean square	F			Odds
			Found	Required		
				5 percent point	1 percent point	
Plants .....	9	639.78	2.80	2.15	2.94	>19:1
Regions .....	5	1,293.60	5.66	2.42	3.45	>99:1
Error .....	45	228.56				
Total .....	59	Standard deviation of the experiment = 15.12.				

## t TEST

Item	Standard error, any mean	Standard error of the difference between any 2 means	Least difference necessary for odds of—	
			19:1	95:1
Plants.....	6.17	8.73	17.58	23.48
Regions.....	4.78	6.76	13.61	18.18

The variance analysis for fiber breaking load in the Rowden 40 variety presented in table 29 indicates very real differences between plants and also between regions.

TABLE 29.—*The analysis of variance of the fiber breaking load by plants and regions on the seed coat of the Rowden 40 variety of cotton*

## REGION MEANS

Region No	1	2	3	4	5	6
Mean breaking load (grams).....	4.30	4.90	4.54	6.72	6.11	6.12

## PLANT MEANS

Plant symbol.....	A	B	C	D	E	F	G	H	I	J
Mean breaking load (grams).....	6.46	4.43	4.38	4.25	7.32	4.28	6.21	4.84	6.25	6.04

## ANALYSIS OF VARIANCE

Variation due to—	Degrees of freedom	Mean square	F			Odds
			Found	Required		
				5 percent point	1 percent point	
Plants.....	9	7.6398	8.46	2.15	2.94	>99:1
Regions.....	5	9.9125	10.98	2.42	3.45	>99:1
Error.....	45	.9028				
Total.....	59	Standard deviation of the experiment = 0.95.				

TABLE 29.—*The analysis of variance of the fiber breaking load by plants and regions on the seed-coat of the Rowden 40 variety of cotton—Continued*

## t TEST

Item	Standard error, any mean	Standard error of the difference between any 2 means	Least difference necessary for odds of—	
			19:1	99:1
Plants .....	0.3878	0.5484	1.104	1.475
Regions .....	.3004	.4248	.856	1.143

■ The data contained in table 30 for the variance of the percentage of thin-walled fibers in the Rowden 40 variety indicate that there are very real differences between regions and nonsignificant differences between plants.

TABLE 30.—*The analysis of variance of the percentage of thin-walled fibers by plants and regions on the seed coat of the Rowden 40 variety of cotton*

## REGION MEANS

Region No. ....	1	2	3	4	5	6
Mean percentage of thin-walled fibers .....	62	30	32	9	9	9

## PLANT MEANS

Plant symbol .....	A	B	C	D	E	F	G	H	I	J
Mean percentage of thin-walled fibers .....	14	21	24	27	25	38	22	36	20	25

## ANALYSIS OF VARIANCE

Variation due to—	Degrees of freedom	Mean square	F			Odds
			Found	Required		
				5 percent point	1 percent point	
Plants .....	9	310.33	1.15	2.15	2.94	<19:1
Regions .....	5	4,441.20	16.52	2.42	3.45	>99:1
Error .....	45	268.91				
Total .....	59	Standard deviation of the experiment = 16.40.				

## t TEST

Item	Standard error, any mean	Standard error of the difference between any 2 means	Least difference necessary for odds of—	
			19:1	99:1
Plants .....	6.69	9.46	19.05	25.45
Regions .....	5.19	7.34	14.78	19.74

The variance analysis for fiber diameter in the Rowden 40 variety is presented in table 31. The values do not point to significant differences between either plants or regions.

#### VARIANCE OF FIBER CHARACTERS FOR THE ENTIRE EXPERIMENT

Results for the analysis of variance within varieties have been given on the preceding pages.

The whole experiment has been set up and analyzed for variance of fiber population, length, weight, breaking load, percentage of thin-walled fibers, and diameter.

TABLE 31.—*The analysis of variance of the fiber diameter by plants and regions on the seed coat in the Rowden 40 variety of cotton*

REGION MEANS										
Region No.....	1	2	3	4	5	6				
Mean fiber diameter (microns).....	16.96	16.52	16.55	16.28	16.78	16.52				
PLANT MEANS										
Plant symbol.....	A	B	C	D	E	F	G	H	I	J
Mean fiber diameter (microns).....	16.53	16.62	16.79	16.32	17.06	16.58	16.10	17.10	16.45	16.45
ANALYSIS OF VARIANCE										
Variation due to—	Degrees of freedom	Mean square	F			Odds				
			Found	Required						
				5 percent point	1 percent point					
Plants.....	9	0.589	1.26	2.15	2.94	<19:1				
Regions.....	5	.560	1.19	2.42	3.45	<19:1				
Error.....	45	.469								
Total.....	59	Standard deviation of the experiment =0.685								
t TEST										
Item	Standard error, any mean	Standard error of the difference between any 2 means	Least difference necessary for odds of—							
			19:1	99:1						
Plants.....	0.280	0.396	0.798	1.065						
Regions.....	.217	.307	.618	.826						

The analysis of variance for the fiber population of the whole experiment is given in table 32, wherein odds of more than 19:1 indicate that there are significant differences between the mean populations of varieties and odds greater than 99:1 show that there are highly significant differences between the mean populations of regions. Odds amounting to more than 19:1 point to the fact that the interac-

tion of varieties with regions (varieties times regions) is significant; that is, varieties significantly affected the fiber population of regions.

TABLE 32.—*The analysis of variance of the fiber population for the total experiment including all five varieties of cotton*

REGION MEANS						
Region No.....	1	2	3	4	5	6
Mean number of fibers.....	1, 673	417	505	253	351	287
VARIETY MEANS						
	Mexican 128	Coker- Cleveland 884-4	Farm Relief No. 1	Acala 4067	Rowden 40	
Mean number of fibers per region .....	529	640	507	662	579	
ANALYSIS OF VARIANCE						
Source of variation	Degrees of freedom	Mean square	F			Odds
			Found	Required		
				5 percent point	1 percent point	
Between varieties.....	4	277, 205	<sup>1</sup> 3.38	2.58	3.77	>19:1
Within varieties.....	45	82, 074				
Between regions.....	5	14, 718, 628	<sup>2</sup> 110.16	2.71	4.10	>99:1
VarietiesXregions.....	20	133, 697	<sup>3</sup> 1.73	1.57	1.88	>19:1
Remainder.....	225	77, 248				
Total.....	299					
t TEST						
Item	Standard error, any mean	Standard error of the difference between any 2 means	Least difference neces- sary for odds of--			
			19:1	99:1		
Varieties <sup>4</sup> .....	37	52	105	140		
Regions.....	52	74	<sup>5</sup> 154	<sup>5</sup> 211		

<sup>1</sup> M. S. varieties

M. S. within varieties

<sup>2</sup> M. S. regions

M. S. varieties×regions

<sup>3</sup> M. S. varieties×regions

M. S. remainder

<sup>4</sup> n for computing standard error any mean=60; n would be 10 if means were plant totals.

<sup>5</sup> Probably not valid for certain comparisons. Least differences calculated from individual standard errors of regions are as follows for these comparisons:

Regions computed	Least differences for odds of—	
	19:1	99:1
2 and 4.....	55	73
2 and 6.....	54	71
3 and 5.....	121	161
4 and 5.....	83	110

The variance analysis of the fiber length for the whole experiment is contained in table 33, wherein odds greater than 99:1 point to the fact that between varieties and also between regions on the seed coat there are highly significant differences. In table 33, also, odds of less than 19:1 indicate that the interaction of varieties and regions is not significant for length; that is, varieties apparently did not significantly affect the fiber length of the regions on the seed.

TABLE 33.—*The analysis of variance of the fiber length of the total experiment including all five varieties of cotton*

REGION MEANS						
Region No. ....	1	2	3	4	5	6
Mean fiber length (inches) .....	1.094	1.097	1.085	1.079	1.044	1.029
VARIETY MEANS						
	Mexican 128	Coker- Cleveland 884-4	Farm Relief No. 1	Acala 4067	Rowden 40	
Mean fiber length per region (inches) .....	1.113	1.102	1.165	0.986	0.991	
ANALYSIS OF VARIANCE						
Source of variation	Degrees of freedom	Mean square	F			Odds
			Found	Required		
				5 percent point	1 percent point	
Between varieties .....	4	0.3801	<sup>1</sup> 11.45	2.58	3.77	>99:1
Within varieties .....	45	.0332				
Between regions .....	5	.0393	<sup>2</sup> 8.93	2.26	3.11	>99:1
Varieties×regions .....	20	.0054	<sup>3</sup> 1.23	1.57	1.88	<19:1
Remainder .....	225	.0044				
Total .....	299					
t TEST						
Item	Standard error any mean	Standard error of the difference between any 2 means	Least difference neces- sary for odds of—			
			19:1	99:1		
Varieties <sup>4</sup> .....	0.0235	0.0333	0.0671	0.0896		
Regions .....	.0094	.0133	.0262	.0346		

<sup>1</sup> M. S. varieties

M. S. within varieties.

<sup>2</sup> M. S. regions

M. S. remainder.

\* Remainder is used as error for regions instead of varieties×regions, which is not significant relative to the remainder.

<sup>3</sup> M. S. varieties × regions

M. S. remainder

<sup>4</sup> n for computing standard error any mean=60; n would be 10 if means were plant totals.



The variance analysis for the fiber weight per inch of the whole experiment is presented in table 34, wherein odds less than 19:1 indicate that the differences between varieties in the fiber weight (average weight of the six regions on the seed coat) are not significant. Odds of greater than 99:1 in table 34 show that differences between regions are highly significant, while odds less than 19:1 point to the fact that varieties did not significantly influence the fiber weight per inch of regions.

TABLE 34.—*The analysis of variance of the fiber weight per inch for the whole experiment including all five varieties of cotton*

REGION MEANS						
Region No	1	2	3	4	5	6
Mean fiber weight (0.0001 mg.)	34	49	44	58	55	59

VARIETY MEANS					
	Mexican 128	Coker- Cleveland 884-4	Farm Relief No. 1	Acala 4067	Rowden 40
Mean fiber weight (0.0001 mg.)	50	47	51	48	53

ANALYSIS OF VARIANCE						
Source of variation	Degrees of freedom	Mean square	F			Odds
			Found	Required		
				5 percent point	1 percent point	
Between varieties	4	309.50	1 0.92	5.63	13.46	<19:1
Within varieties	45	335.38				
Between regions	5	4,523.00	2 56.88	2.26	3.11	>99:1
Varieties×regions	20	93.50	3 1.18	1.57	1.88	<19:1
Remainder	225	79.52				
Total	299					

t TEST				
Item	Standard error, any mean	Standard error of the difference between any 2 means	Least difference neces- sary for odds of—	
			19:1	99:1
Varieties <sup>1</sup>	2.363	3.342	6.731	8.990
Regions	1.263	1.784	3.518	4.640

<sup>1</sup> M. S. varieties

M. S. within varieties.

<sup>2</sup> M. S. regions

M. S. remainder. Remainder is used as error for regions instead of varieties×regions, which is not significant relative to the remainder.

<sup>3</sup> M. S. varieties × regions.

M. S. remainder

<sup>4</sup> n for computing standard error any mean=60; n would be 10 if means were plant totals.

The variance analysis for the single fiber breaking load of the entire experiment is contained in table 35, wherein odds greater than 19:1 indicate significant differences between varieties. Odds of more than 99:1 show that there are highly significant differences between region means, while odds less than 19:1 point to the fact that varieties did not significantly influence the fiber breaking load of regions.

TABLE 35.—*The analysis of variance of the fiber breaking load for the total experiment including all five varieties of cotton*

REGION MEANS						
Region No	1	2	3	4	5	6
Mean breaking load (grams)	3.44	4.73	4.32	6.11	5.70	5.78

VARIETY MEANS					
	Mexican 128	Coker- Cleveland 884-4	Farm Relief No. 1	Acala 4067	Rowden 40
Mean fiber breaking load (grams)	5.42	4.54	4.75	4.92	5.45

ANALYSIS OF VARIANCE						
Source of variation	Degrees of freedom	Mean square	F			Odds
			Found	Required		
				5 percent point	1 percent point	
Between varieties	4	9.8257	13.56	2.58	3.77	>19:1
Within varieties	45	2.7567				
Between regions	5	53.2078	285.06	2.26	3.11	>99:1
Varieties×regions	20	.6044	3.97	1.84	2.42	<19:1
Remainder	225	.6255				
Total	299					

t TEST				
Item	Standard error, any mean	Standard error of the difference between any 2 means	Least difference neces- sary for odds of—	
			19:1	99:1
Varieties <sup>4</sup>	0.2142	0.3029	0.6100	0.8148
Regions	.1119	.1582	.3120	.4115

<sup>1</sup> M. S. varieties

M. S. within varieties

<sup>2</sup> M. S. Regions

M. S. remainder. Remainder is used as error for regions instead of varieties×regions, which is not significant relative to the remainder.

<sup>3</sup> M. S. varieties×regions.

M. S. remainder

<sup>4</sup> *n* for computing standard error any mean=60; *n* would be 10 if means were plant totals.

The analysis of variance of the percentage of thin-walled fibers for the total experiment, presented in table 36, gives odds greater than 19:1 for varieties and odds greater than 99:1 for regions, and also for

varieties  $\times$  regions; that is, there are differences between both varieties and regions, and varieties significantly affected regions relative to the percentage of thin-walled fibers.

TABLE 36.—*The analysis of variance of the percentage of thin-walled fibers for the total experiment including all five varieties of cotton*

REGION MEANS						
Region No. ....	1	2	3	4	5	6
Mean percentage of thin-walled fibers.....	50	19	28	8	9	8

VARIETY MEANS					
	Mexican 128	Coker- Cleveland 884-4	Farm Relief No. 1	Acala 4067	Rowden 40
Mean percentage of thin-walled fibers....	16	18	20	23	25

ANALYSIS OF VARIANCE						
Source of variation	Degrees of freedom	Mean square	F			Odds
			Found	Required		
				5 percent point	1 percent point	
Between varieties	4	836.96	1 2.61	2.58	3.77	>19:1
Within varieties	45	320.67				
Between regions	5	13,933.49	2 33.66	2.71	4.10	>99:1
Varieties×Regions	20	413.94	3 2.63	1.57	1.88	>99:1
Remainder	225	157.41				
Total	299					

#### t TEST

Item	Standard error, any mean	Standard error of the difference between any 2 means	Least difference neces- sary for odds of—	
			19:1	99:1
Varieties.....	2.311	3.268	6.58	8.79
Regions.....	2.877	4.069	8.49	11.58

<sup>1</sup> M. S. varieties

M. S. within varieties

<sup>2</sup> M. S. regions

M. S. varieties  $\times$  regions

<sup>3</sup> M. S. varieties  $\times$  regions

M. S. remainder

<sup>4</sup> *n* for computing standard error, any mean = 60; *n* would be 10 if means were plant totals.

The analysis of variance of the fiber diameter for the entire experiment is given in table 37. Odds greater than 99:1 in this table indicate that there are highly significant differences between varieties and also point to the fact that varieties very significantly influenced the fiber diameter of regions. Odds of less than 19:1 in the same table indicate that differences between regions are not significant.

TABLE 37.—*The analysis of variance of the fiber diameter for the total experiment including all five varieties of cotton*

REGION MEANS						
Region No	1	2	3	4	5	6
Mean fiber diameter (microns)	16.40	16.18	16.12	15.90	15.99	16.00

VARIETY MEANS					
	Mexican 128	Coker- Cleveland 884-4	Farm Relief No. 1	Acala 4067	Rowden 40
Mean fiber diameter (microns)	16.32	15.62	16.85	15.09	16.60

Source of variation	Degrees of freedom	Mean square	F			Odds
			Found	Required		
				5 percent point	1 percent point	
Between varieties	4	31.81	<sup>1</sup> 13.37	2.58	3.77	>99:1
Within varieties	45	2.38				
Between regions	5	1.59	<sup>2</sup> 1.36	2.71	4.10	<10:1
Varieties×regions	20	1.17	<sup>3</sup> 2.05	1.57	1.88	>99:1
Remainder	225	.57				
Total	290					

## t TEST

Item	Standard error, any mean	Standard error of the difference between any 2 means	Least difference neces- sary for odds of—	
			19:1	99:1
Varities <sup>4</sup>	0.1991	0.2816	0.57	0.76
Regions	.1530	.2164	.45	.62

<sup>1</sup> M. S. varieties

M. S. within varieties

<sup>2</sup> M. S. regions

M. S. varieties×regions

<sup>3</sup> M. S. varieties×regions

M. S. remainder

<sup>4</sup> n for computing standard error any mean=60; n would be 10 if means were plant totals.

## COMPARISON OF THE FIBER POPULATION OF VARIETIES BY SINGLE REGIONS

From data on the fiber population of each of the six regions from the 10 plants of each of the five varieties calculations were made to obtain the analysis of variance values for population. These summarized values are presented in table 38. Between varieties the odds shown are less than 19:1 for regions 1, 3, and 4, 19:1 for region 2, and greater than 99:1 for regions 5 and 6.

TABLE 38.—The variance of fiber population by single regions for between the five varieties of cotton

## OBSERVED VALUES

Varieties	Mean fiber population for region No.—					
	1	2	3	4	5	6
Mexican 128.....	1,457	296	476	286	327	317
Coker-Cleveland 884-4.....	2,044	437	513	273	334	241
Farm Relief No. 1.....	1,493	440	361	233	261	256
Acala 4067.....	1,646	499	753	270	395	406
Rowden 40.....	1,725	414	424	202	438	216

## ANALYSIS OF VARIANCE

Region No.---	Variation due to---	Degrees of free- dom	Mean square	F			Odds
				Found	Required		
					5 percent point	1 percent point	
1	Varieties.....	4	549,268	2.31	2.58	2.77	<19:1
	Error.....	45	239,022				
	Total.....	49					
2	Varieties.....	4	55,702	2.582	2.58	3.77	19:1
	Error.....	45	21,575				
	Total.....	49					
3	Varieties.....	4	224,246	2.06	2.58	3.77	<19:1
	Error.....	45	109,087				
	Total.....	49					
4	Varieties.....	4	11,843	.77	5.63	13.46	<19:1
	Error.....	45	15,380				
	Total.....	49					
5	Varieties.....	4	682,924	34.95	2.58	3.77	>99:1
	Error.....	45	19,539				
	Total.....	49					
6	Varieties.....	4	57,931	7.17	2.58	3.77	>99:1
	Error.....	45	8,085				
	Total.....	49					

## t TEST

Region No.—	Standard error, any mean	Standard error of the difference between any 2 means	Least difference necessary for odds of—	
			19:1	99:1
1.....	154	218	439	596
2.....	46	65	131	175
3.....	105	148	298	398
4.....	39	55	111	148
5.....	44	63	127	169
6.....	29	49	81	108

## COMPARISON OF VARIETIES FOR GOODNESS OF FIT FOR FIBER DISTRIBUTION

The value of  $\chi^2$  was determined for each of 10 possible pairs of varieties from the formula <sup>10</sup>

$$\chi^2 = \left[ \sum \frac{(f_a/N_a - f_b/N_b)^2}{f_a + f_b} \right] N_a N_b$$

in which  $f_a$  and  $f_b$  are the frequencies and  $N_a$  and  $N_b$  are the totals of these frequencies. The distribution of the fiber population,  $\chi^2$ , and  $P$  values are presented in table 39. The  $P$  values in this table are less than 0.000005 and the odds greater than 199,999:1 for any pair of varieties; therefore the distribution of fiber population is different in each possible comparison.

TABLE 39.—The goodness of fit for the fiber distribution of all five cotton varieties

Region No. or class <sup>1</sup>	OBSERVED VALUES				
	Average fiber population of variety named—				
	Mexican 128	Coker-Cleveland 884-4	Farm Relief No. 1	Acala 4067	Rowden 40
1 .....	1,457	2,044	1,493	1,646	1,725
2 .....	206	457	440	499	414
3 .....	477	513	361	753	424
4 .....	286	273	233	270	202
5 .....	327	334	261	305	438
6 .....	317	211	256	406	216

χ <sup>2</sup> VALUE			
Varities compared	Value of χ <sup>2</sup>	Value of P	Odds
Mexican 128 and Coker-Cleveland 884 4 .....	71.87	0.000065	Greater than 199,999:1.
Mexican 128 and Farm Relief No. 1 .....	61.75		
Mexican 128 and Acala 4067 .....	51.92		
Mexican 128 and Rowden 40 .....	84.70		
Coker-Cleveland 884-4 and Farm Relief No. 1 .....	32.75		
Coker-Cleveland 884-4 and Acala 4067 .....	137.70		
Coker-Cleveland 884-4 and Rowden 40 .....	37.57		
Farm Relief No. 1 and Acala 4067 .....	92.79		
Farm Relief No. 1 and Rowden 40 .....	51.41		
Acala 4067 and Rowden 40 .....	131.49		

<sup>1</sup>  $n' = 6$  regions or classes. Then  $n$  for value of  $P$  in Elderton's table (where  $n' = 5 + 1 = 6$ ) equals 5. (See ELDERTON, W. PALIN. TABLES FOR TESTING THE GOODNESS OF FIT OF THEORY TO OBSERVATION. Biometrika 1:[155]-163. 1901.) One degree of freedom was used in the classification for the 6 regions.

## SIMPLE CORRELATIONS WITHIN VARIETIES

From the data in columns 2 to 7, lines 1 to 10, of tables 2, 3, 4, 5, 6, and 7 simple correlation values were calculated to show the relationship of density of fiber population, fiber length, fiber weight, fiber strength, percentage of thin-walled fibers, and fiber diameter in the Mexican 128 variety of cotton. Calculations were also made from similar data for the other four varieties to obtain this relationship. The correlation coefficients for the five varieties are presented in table 40.

<sup>10</sup> LOVE, HARRY H. APPLICATION OF STATISTICAL METHODS TO AGRICULTURAL RESEARCH. 501 pp., illus. Shanghai. 1937. (See pp. 293-296.)

The values in table 40 are not significant for the correlation of density of fiber population with fiber length; whereas all the values for the correlation of density of fiber population with fiber weight are highly significant and negative—that is, as fiber population increases fiber weight decreases, or vice versa. The correlation of density of fiber population with the average strength per fiber is also negative and highly significant for each variety. The correlation values indicate a highly significant and positive relationship of density of fiber population with the percentage of thin-walled fibers in each variety. No variety shows a significant value for the relation of density of fiber population to the average fiber diameter.

TABLE 40.—*The simple correlation within varieties of density of fiber population, fiber length, fiber weight, fiber strength, percentage of thin-walled fibers, and fiber diameter for all five varieties of cotton.*

Variables paired	Simple correlation coefficients for variety named <sup>1</sup>				
	Mexican 128	Coker-Cleveland 884-4	Farm Relief No. 1	Acala 4067	Rowden 40
Density of fiber population and average fiber length	0.0780	0.1164	-.0.1528	0.2200	0.2354
Density of fiber population and average fiber weight per inch	**-.6745	**-.7089	**-.6800	**-.6978	**-.5413
Density of fiber population and average strength per fiber	**-.5582	**-.7152	**-.6771	**-.6215	**-.4270
Density of fiber population and percentage of thin-walled fibers	**-.7088	**-.6361	**-.7830	**-.7741	**-.6406
Density of fiber population and average fiber diameter	-.1083	-.0741	.2288	.0826	.2439
Average fiber length and average fiber weight per inch	**-.4106	**-.3853	-.1562	**-.4443	-.2495
Average fiber length and average fiber strength	-.0732	-.0379	.0339	*-.2548	-.0758
Average fiber length and percentage of thin-walled fibers	.1076	.1338	.0454	**-.3467	*.2876
Average fiber length and average fiber diameter	**-.3433	-.1894	-.1702	.0705	.1612
Average fiber weight per inch and average fiber strength	**-.6281	**-.7922	**-.5728	**-.7972	**-.3832
Average fiber weight per inch and percentage of thin-walled fibers	**-.5919	**-.7202	**-.7928	**-.7725	**-.4416
Average fiber weight per inch and average fiber diameter	**-.3561	.2006	-.0743	.1539	.0985
Average fiber strength and percentage of thin-walled fibers	**-.5831	**-.7360	**-.5754	**-.7751	**-.7717
Average fiber strength and average diameter	.0856	.1187	*-.2955	-.0636	-.0144
Percentage of thin-walled fibers and average diameter	-.0001	-.0744	*.2585	.0169	*.2740

<sup>1</sup>  $n = 60$  regions.  $n - 2 = 58$  degrees of freedom for each variety. \*significant values; \*\*highly significant values. Significance in this and all other correlation tables of this paper is based upon the required values given by WALLACE, H. A., and SNEDECOR, GEORGE W. CORRELATION AND MACHINE CALCULATION. Rev. by G. W. Snedecor. Iowa State Col. Off. Pub. 30, No. 4, 71 pp., illus. 1931. (See table 16.)

The correlation coefficients in table 40 for the relation of average fiber length to average fiber weight per inch are all negative, but are significant in only three varieties; namely, Mexican 128, Coker-Cleveland 884-4, and Acala 4067. The values show that the correlation of the average fiber length with the average fiber strength is significant in only one variety, Acala 4067, for which the value is also negative. The values for the relationship of average fiber length to the percentage of thin-walled fibers are all positive, but they are significant in only two varieties; namely, Acala 4067 and Rowden 40. The correlation of average fiber length with average fiber diameter is significant in only one variety, Mexican 128, for which it is negative.

The coefficients for the relationship of average fiber weight and average fiber strength in table 40 are all positive and highly significant. The values for the correlation of fiber weight with the percentage of thin-walled fibers are negative and highly significant for each variety. The values indicate that the correlation of fiber weight with fiber diameter is significant in only one variety, Mexican 128, for which it is positive.

The values in table 40 show a highly significant and negative correlation of average fiber strength with the percentage of thin-walled fibers in each variety. The correlation of fiber strength and average fiber diameter is significant in only one variety, Farm Relief No. 1, in which the association is negative.

The coefficients given in table 40 for the relation of the percentage of thin-walled fibers to the average fiber diameter are significant in only two varieties, Farm Relief No. 1 and Rowden 40. The association is positive.

#### SIMPLE AND PARTIAL CORRELATIONS FOR THE ENTIRE EXPERIMENT

Simple and partial correlations of the fiber characters by regions on the cottonseed and also by plants, or seeds, are shown for the entire experiment in table 41.

#### CORRELATIONS BY REGIONS

The correlation coefficients calculated for any character from the 300 regions on the 50 seeds of the five cotton varieties ( $6 \text{ regions} \times 50 \text{ seeds} = 300 \text{ regions}$ ) are based upon differences between regions within seeds and also between regions of seeds, or plants. The values in table 41 calculated from 300 regions are therefore not independent of the position of the fibers on the seed coat. The simple and partial correlations calculated from the data of 300 regions and shown in table 41 indicate the following relationships:

(1) The simple association of fiber population with average fiber length is not significant, while the partial association is significant and negative. As density of fiber population increases, fiber length decreases when the other characters, fiber weight, fiber strength, percentage of thin-walled fibers, and fiber diameter, are held constant. The simple and partial values for the relationship of fiber population with average fiber weight per inch, or unit weight, and also with average breaking load, or strength, per fiber are very significant and also negative. Positive and highly significant values are recorded for the simple and partial correlations of fiber population with the percentage of thin-walled fibers; that is, an increase in density of fiber population is associated with an increase in the percentage of thin-walled fibers. The simple and partial values for the association of fiber population with average fiber diameter are not significant.

(2) The simple and partial values indicate a negative and very real correlation of average fiber length with fiber weight per inch. The simple correlation of fiber length with fiber strength is significant with a negative value, while the partial relationship is not significant. Neither the simple nor the partial value for the relation of fiber length and the percentage of thin-walled fibers is significant. The coefficient for the simple correlation of fiber length and diameter is not considered significant, but the partial value for this relationship is positive and highly significant.



TABLE 41.—Simple and partial correlations of density of fiber population, fiber length, fiber weight, fiber strength, percentage of thin-walled fibers, and fiber diameter for the entire experiment including all five varieties of cotton

Simple order subscript <sup>1</sup>	Simple correlation coefficients <sup>2</sup>		Partial order subscript	Partial order coefficients	
	By regions (n' = 300) <sup>3</sup>	By plants, or seeds (n' = 50) <sup>4</sup>		By regions (n' = 300) <sup>3</sup>	By plants, or seeds (n' = 50) <sup>4</sup>
12.....	0.0515	*-0.2936	12.3456	*-0.1235	*-0.3071
13.....	**-.6312	*-.2827	13.2456	**-.3373	—, 1895
14.....	**-.5662	—, 2276	14.2356	**-.1798	—, 1750
15.....	**-.6601	.2643	15.2346	**-.3661	.2081
16.....	—, 0138	**-.4310	16.2345	.0437	.2440
23.....	**-.2561	—, 2455	23.1456	**-.3022	**-.4237
24.....	*-.1245	—, 1604	24.1356	—, 0221	—, 2654
25.....	.0762	—, 1006	25.1346	—, 0710	—, 1250
26.....	.0811	.1864	26.1345	**-.1604	.2845
34.....	**-.5874	.1730	34.1256	**-.2000	—, 0600
35.....	**-.5924	—, 1143	35.1246	**-.2562	—, 1330
36.....	**-.1801	**-.4544	36.1245	**-.2975	**-.4532
45.....	**-.5799	—, 1800	45.1236	**-.2473	—, 1730
46.....	.0311	.2105	46.1235	—, 0017	.1800
56.....	.0824	—, 0058	56.1234	**-.2037	.1940

<sup>1</sup> 1=density of fiber population; 2=average fiber length; 3=average fiber weight per inch; 4=average breaking load per fiber; 5=percentage of thin-walled fibers; 6=average fiber diameter.

<sup>2</sup> \*Significant value; \*\*highly significant value.

<sup>3</sup> n' = 300 regions. n' - 2 = 298 degrees of freedom for each simple value. n' - 6 = 294 degrees of freedom for each partial value.

<sup>4</sup> n' = 50 plants, or seeds. n' - 2 = 48 degrees of freedom for each simple value. n' - 6 = 44 degrees of freedom for each partial value.

(3) The simple correlation of average fiber weight with average fiber strength is positive and very significant, and the same is true of the partial relationship. Negative and highly significant simple and partial coefficients are shown for the relation of fiber weight and the percentage of thin-walled fibers. Positive and very significant simple and partial values are noted for the correlation of fiber weight and average fiber diameter.

(4) Negative and highly significant simple and partial correlations are recorded for the association of average fiber strength and the percentage of thin-walled fibers. Simple and partial values for the relationship of fiber strength and fiber diameter are not considered significant.

(5) The value for the simple correlation of the percentage of thin-walled fibers with average fiber diameter is not noted as significant, while the partial value is positive and very real.

#### CORRELATIONS BY PLANTS, OR SEEDS

The mean value of any fiber character for a cottonseed, or plant, was obtained by summing the values of the six regions and then dividing the total by six. This mean value represents the average value of the six regions but does not necessarily represent the true average of any of the fiber characters on any one seed, or plant. The correlation coefficients in table 41 have been calculated from 50 plant, or seed, means for each character, and therefore the influence of position on the seed upon any fiber character has been eliminated in the correlation values. The calculations are based upon differences between plants within varieties and also upon differences between plants of different varieties, and not upon differences between regions, or positions. The simple and partial correlation coefficients calculated

from the mean values of 50 plants, or seeds, and presented in table 41 point to the following relationships:

(1) The association of fiber population and fiber length is indicated by significant and negative values for both the simple and the partial orders. The simple correlation value for the relation of fiber population to unit fiber weight is significant and negative, while the partial value is not indicated as significant. The simple and partial values for the association of fiber population with fiber strength and also with the percentage of thin-walled fibers do not appear to be significant. The simple correlation of fiber population and fiber diameter is highly significant and negative, but the partial value is not recorded as significant.

(2) The total correlation of fiber length with unit fiber weight is not significant, but a highly significant and negative value is indicated for the partial order. None of the simple or partial order values are significant for the correlation of fiber length with either fiber strength or percentage of thin-walled fibers. The simple association of fiber length and fiber diameter does not appear to be significant, and the partial value is not significant.

(3) The simple and partial values do not indicate a significant correlation of fiber weight with either fiber strength or percentage of thin-walled fibers. The association of fiber weight and fiber diameter is positive and very real in both the simple and the partial relationships.

(4) No significant relationship of fiber strength with either the percentage of thin-walled fibers or the fiber diameter is indicated by the simple and partial values.

(5) The simple and partial order values do not indicate a significant association of the percentage of thin-walled fibers and fiber diameter.

## DISCUSSION

The results presented show that for each of the five varieties the population of region 1 (which includes the chalazal area at the basal end of the seed) is significantly denser than that of any other region. The mean fiber population for each of the six regions (see fig. 1) studied indicates that the varieties are somewhat similar in the distribution of fiber population, in that density is highest at the chalazal region and gradually becomes less as the raphe and the micropylar end of the seed are approached. The mean fiber population for regions, or punch areas, of the whole experiment indicates that the population of region 1 is significantly denser than that of any other region; that the population of region 3 is significantly denser than that of regions 4, 5, and 6; that the population of region 2 is significantly denser than that of regions 4 and 6; and that the population of region 5 is significantly denser than that of region 4. There is apparently a tendency for the fiber population to decrease in going from the chalazal area toward the micropylar, or apical, end of the seed; and the population also appears to decrease in a horizontal direction (see fig. 1) in going from regions 3, 5, and 6, toward the raphe. In order to compare these results with those of previous work, the fiber-population means per region, or punch area, in table 32 have been divided by the area per region, 3.567 mm.<sup>2</sup>, and are presented in table 42, along with data previously obtained.

TABLE 42.—*The fiber distribution of the entire experiment as compared with that obtained from previous data*

OBSERVED VALUES			
Region No. <sup>1</sup>	Source of data and average fiber population per square millimeter of seed-coat surface		
	Entire experiment consisting of 5 varieties (1937 crop)	20 naked <sup>2</sup> seeds from 20 plants (1924 crop) <sup>3</sup>	4 seeds <sup>4</sup> from 4 plants (1924 crop) <sup>3</sup>
1	469	175	210
2	117	57	81
3	142	64	116
4	71	32	47
5	98	43	78
6	81	16	49
VALUE			
Kind of comparison	Value of $\chi^2$	Value of $P$	Odds
Columns 2 and 3 from above	8.12	0.087847	10 to 1
Columns 2 and 4 from above	20.67	.000257	3,890 to 1

<sup>1</sup>  $n'=6$  regions. Then  $n$  for value of  $P$  in Elderton's table (where  $n'=5+1=6$ ) equals 5. 1 degree of freedom was used in the classification for the 6 regions. (See reference given in footnote to table 39, p. 293.)

<sup>2</sup> No fuzz on seeds and also a sparse fiber population.

<sup>3</sup> See footnote, 256.

<sup>4</sup> Fuzzy tip and base and sparse fuzz on middle part of seed. Normal fiber population.

The values for goodness of fit in table 42 show that there is fair agreement for the fiber distributions of the present results with the data obtained <sup>11</sup> on fuzzless seeds. The agreement is poor, however, when the present data are compared with results from seeds having dense fuzz on the tip and base and sparse fuzz on the middle portion of the seed coat. The data in columns 2, 3, and 4 show a similarity in the three sets of data; namely, in each case the population is densest on region 1 and tends gradually to decrease in areas toward the micropylar end and the raphe of the seed.

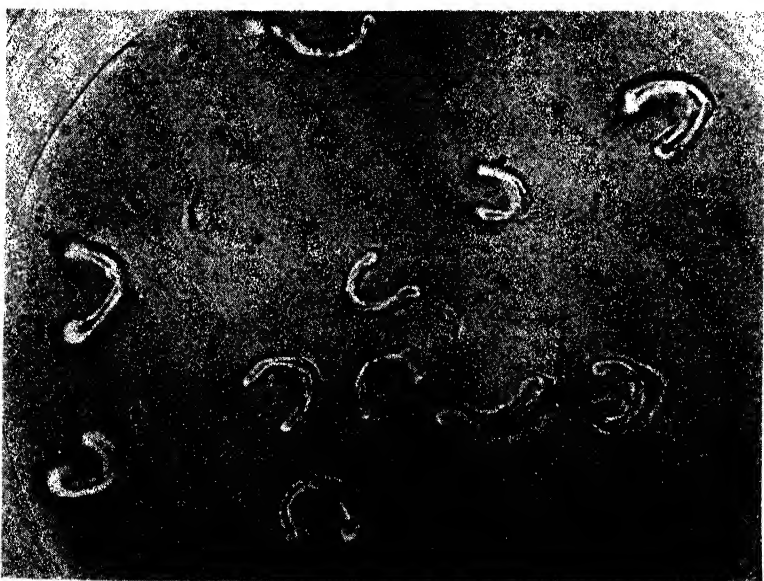
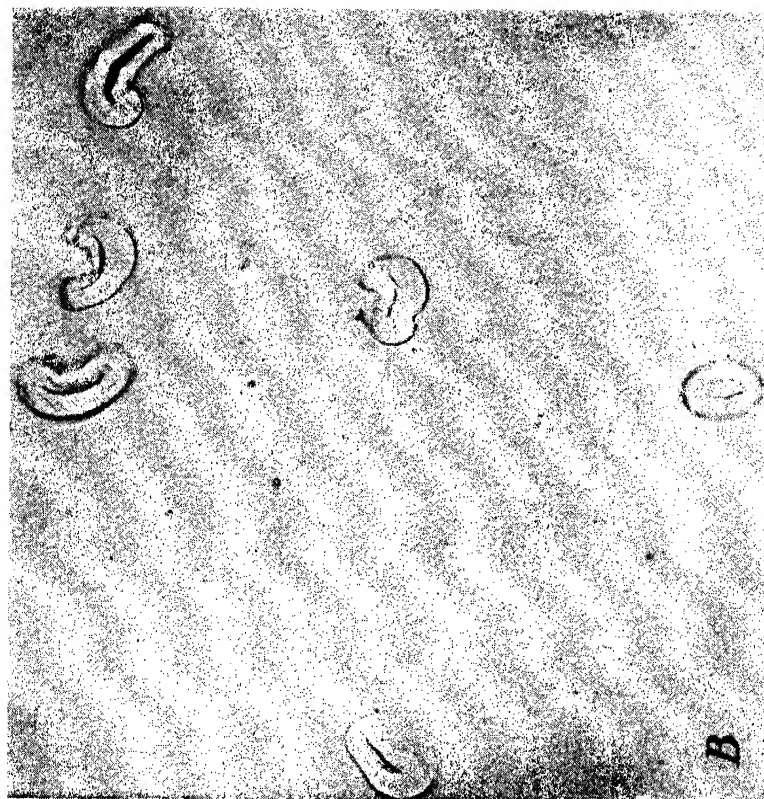
The average fiber length of regions for the whole experiment indicates that the length from basal regions (1, 2, and 3) is greater than the length of regions (4, 5, and 6) near the apical, or pointed, end of the seed. This agrees with the report made by Koshal and Ahmad <sup>12</sup> on Surat and Indian Standard cottons. They state that the mean fiber length at the base of the seed is significantly longer than that at the apex of the seed.

The average fiber weight by varieties and for the whole experiment shows that the weight per inch of fibers from region 1 is much lighter than that from any other region. The results from the same source also indicate a lower fiber weight for the basal area (regions 1, 2, and 3) than for the area (regions 3, 4, and 5) near the apex, or tip, of the seed. Such conclusions are also similar to those published by Koshal and Ahmad, who report that the mean fiber weight per unit of length is significantly higher for the apical than for the basal fibers. Campbell <sup>13</sup> also found that the percentage of undeveloped fibers (having thin walls and relatively light weight) is highest at the basal end of the seed, becoming lower as the pointed end is approached.

<sup>11</sup> See footnote 2.

<sup>12</sup> See footnote 4.

<sup>13</sup> See footnote 3.



Photomicrographs showing unstained transsections of mature cotton fibers from regions 1 and 6 of a seed from one of the cotton varieties studied: *A*, Relatively thin fiber walls from region 1, which includes the chalazal area; *B*, relatively thick fiber walls from region 6, near the micropylar end.  $\times 528$ .



Photomicrographs of longisections from a portion of the seed coat from a mature seed of one of the cotton varieties studied:  
*A*, From chalazal area; shows a relatively large amount of intercellular space between the vein and the epidermal layer, to which are attached portions of three cotton hairs. *B*, From approximately region 3 and some distance from the chalaza; shows a relatively small amount of intercellular space between the vein and the epidermal layer, to which are attached portions of two cotton hairs; parts of hairs which are not attached to the epidermal layer and cut at various angles are also shown.  $\times 316$ .

Within each of the five varieties and for the entire experiment, the average breaking load of fibers from the basal part of the seed is significantly lower than that of fibers from the apical portion of the seed. Within varieties (with the exception of Rowden 40) and for the whole experiment, the average breaking load of fibers from region 1 is significantly less than that of any other region. Similar results are reported by Koshal and Ahmad, who measured the breaking load of single fibers at and near the chalazal region and also at the apical end of the seed and who state that the mean fiber strength is significantly higher for the apical than for the basal fibers in Surat and Indian Standard cottons.

Within varieties and also for the entire experiment the percentage of thin-walled fibers is much higher on region 1 than on any other region. For the entire experiment the percentage of thin-walled fibers is significantly higher on the basal end of the seed than on the apical end; and within varieties the condition is similar. Transections of mature cotton fibers from regions 1 and 6 of a cotton seed are shown in plate 1. It will be noted that the thin-walled fibers are predominant in region 1 and very scarce in region 6. Campbell has already been quoted in the discussion of fiber weight.

Significant differences in fiber diameter between regions on the seed coat within varieties are recorded for only two varieties, Acala 4067 and Mexican 128. For the entire experiment the results indicate no significant differences in the average fiber diameter between regions on the seed coat. The detailed records on file point to the fact that variability in the fiber diameter of the five cotton varieties is due mostly to variability within regions.

The distribution of each of the six fiber characters on the cottonseed has been discussed and compared with previous data, but no reasons have been advanced to explain the distributions. Why is the fiber population denser on the basal surface of the seed, especially in the chalazal area, than on surfaces nearer the raphe and the tip, or micropylar end? The fiber population on the seed coat of naked seed and other types was previously determined by the author, who also made studies relative to the venation of the seed coat. A conclusion was reached that the venation of the seed coat was relatively sparse in the areas bordering each side of the tip half of the raphe and that a relatively low fiber population occurred in such areas. The venation pattern may be related to the supply of food and thus to the variability of density of population; but, since density of population and unit weight are inversely correlated, there is not much, if any, evidence for the first relationship. The fiber population in American upland cotton types is determined within about 3 days (counting the date of flowering as the first day); the maximum fiber diameter is probably attained by the third day, and the full fiber length is reached in from 17 to 20 days, after which growth of the secondary wall begins and continues for an indefinite number of days until it is completed. It does not seem that differences in the available food supply would affect the density of fiber population, since it is likely that there is sufficient food available at any location on the seed coat for the production of hair initials. Another question might also be asked. Why are the longer, weaker, and lighter fibers (per unit of length) with a relatively high percentage of thin walls on the basal surface of the seed? The available food supply as affected by variation in venation

does not answer this question. The relatively shorter fibers near the tip end of the seed are also heavier per unit of length, and it is probable that much more food is used for cell thickening than for elongation. Since the fibers at and near the apex of the seed coat are heavier, they also have thicker cellulose walls and are stronger.

It is conceivable that the very low relative fiber weight of region 1, which includes the chalazal area, is related to the loose arrangement of cells under the epidermal layer. That is, there is an appreciable area of space between the cells (pl. 2), and as a result transportation of foods to the chalazal area may be adversely affected. Since this region also has a high population, the limited food supply may not be sufficient for the normal wall development of all the fibers. Such a condition could be responsible for the relatively low fiber weight, relatively low fiber strength, and relatively high percentage of thin-walled fibers of region 1. No satisfactory explanation can be given to answer the two questions that have been raised. It is probable that inherent factors and physiology both play an important role in determining the distribution of fiber population, length, unit weight, strength, percentage of thin-walled fibers, and diameter.

Differences between plants and also between varieties in the distribution of fiber population, length, unit weight, strength, percentage of thin-walled fibers, and diameter are probably inherent.

#### SUMMARY AND CONCLUSIONS

The distribution and relation of fiber population, length, breaking load, weight, diameter, and percentage of thin-walled fibers on the cottonseed were measured in the 1937 crop of five varieties of American upland cotton (*Gossypium hirsutum* L.). Six regions, or punch areas, numbered one to six, were studied on each seed. Ten seeds from ten plants of each variety were investigated.

Analysis of variance was applied to the data for each variety and also to the data for the entire experiment. Simple correlation coefficients were calculated to show the relationship of the six fiber characters one to another within varieties, and both simple and partial correlation values were obtained to indicate their relationship for the entire experiment by regions, or punch areas, and also by seeds, or plants.

The fiber population is densest at and near the chalazal area of the seed and becomes thinner downward toward the micropylar end and outward toward the raphe. Goodness of fit between the five varieties for the fiber population of the six regions shows that no two varieties are alike in the fiber distribution.

The results from the whole experiment indicate that fibers on the basal end of the seed are lighter, thinner, weaker, and longer than those on the apical or micropylar end.

Varieties significantly affect the population, diameter, and percentage of thin-walled fibers of regions.

There are real differences between varieties in the means of fiber population, length, breaking load, diameter, and percentage of thin-walled fibers.

The results obtained should serve as stepping stones to further progress in cotton-breeding and production work. Plants (as represented by one seed) within a variety show differences among themselves relative to fiber length, unit fiber weight, fiber strength, fiber

diameter, and percentage of thin-walled fibers—that is, regional differences. Some plants are more uniform than others. In the five varieties studied, however, it seems that the variation of fiber length and diameter on the seed is due mostly to variability within regions. This indicates that selection of plants for uniformity of length and diameter should be largely on the basis of uniformity of the whole fiber population of the seed without especial regard to regional differences. It appears that the breeder may be able to select plants which show a relatively low amount of variation in fiber weight, fiber strength, and percentage of thin-walled fibers between regions on the seed and thus eliminate some of the variability in these properties.

The present paper suggests possibilities in cotton improvement work; yet it, along with records on file, also indicates that there is probably considerable variation between regions in the fiber population, fiber length, unit fiber weight, fiber strength, and percentage of thin-walled fibers on single seeds of the five improved cotton varieties, indicating that the uniformity of a variety in any of these characters is limited by the variability found on the seed. Significant differences between varieties in the distribution and means of fiber population, length, strength, percentage of thin-walled fibers, and diameter are probably inherent. Differences between varieties in fiber length are of some value in varietal classification, and it is conceivable that differences between them in the distribution of fiber population may help in taxonomic classification.

The simple correlation within varieties of density of fiber population with average fiber weight per inch, with average strength per fiber, and with the percentage of thin-walled fibers is very significant and striking in each variety; and an increasing fiber population is thus associated with a decreasing fiber weight and fiber strength and with an increasing percentage of thin-walled fibers. Within each variety the simple correlations indicate a very consistent association of average fiber weight per inch with average strength per fiber and also with the average percentage of thin-walled fibers. An increasing fiber weight is correlated with an increasing fiber strength and with a decreasing percentage of thin-walled fibers. All these relationships, although not independent of the position of the fibers on the seed and the other fiber characters involved, appear to have some significance. For instance, selection for a denser fiber population should also lower both the fiber weight and the fiber strength and should raise the percentage of thin-walled fibers. Selection for a higher fiber weight per inch should raise the fiber strength and lower the percentage of thin-walled fibers. The simple correlation of average strength per fiber with the percentage of thin-walled fibers is very consistent and significant within each variety, where an increasing fiber strength is associated with a decreasing percentage of thin-walled fibers.

The simple and partial correlations by regions for the entire experiment of density of fiber population with average fiber weight per inch, with average strength per fiber, and with the percentage of thin-walled fibers are similar in each relationship to the simple values within varieties; that is, an increasing population is associated with a decreasing fiber weight and fiber strength and with an increasing percentage of thin-walled fibers. For the entire experiment by regions the total, or simple, correlation of fiber population with fiber length



does not appear to be significant, while the partial value is significant and negative; that is, with fiber weight, strength, diameter, and the percentage of thin-walled fibers held constant, an increasing fiber population is associated with a decreasing fiber length. The partial relationships discussed in this paragraph are not independent of the position of fibers upon the seed; however, it appears that selection on the cottonseed for a higher population should decrease fiber length, weight, and strength and increase the percentage of thin-walled fibers. For the whole experiment by regions there is a real negative association of average fiber length with average fiber weight per inch in both the total and the partial orders; the former fiber character shows a significant and negative simple association with average strength per fiber, but the partial value is very small and not significant; and independent of all other fiber characters there is perhaps a positive association of fiber length with average fiber diameter. Raising the fiber length should therefore theoretically increase fiber diameter and lower fiber weight. For the entire experiment by regions average fiber weight per inch appears to be correlated with average strength per fiber, with the percentage of thin-walled fibers, and with average fiber diameter in both the simple and the partial orders; that is, as fiber weight goes up, fiber strength and diameter increase and the percentage of thin-walled fibers decreases. As average strength per fiber for the whole experiment by regions increases, the percentage of thin-walled fibers goes down in both the simple and the partial orders; and an increase in the percentage of thin-walled fibers is associated with a larger fiber diameter in the partial relationship.

In a general way the total and partial-order correlations for the complete experiment by plants, or seeds, are similar to those for the entire experiment by regions, especially as to signs; but relatively few of the values are significant. This might be expected since there are only 50 plants, whereas there are 300 regions. For the entire experiment by plants (where each plant, or seed, is an average of the 6 regions and where the differences are between plants and not between regions, or position) density of fiber population is negatively associated with average fiber length in both the simple and the partial orders, and a negative relationship is also noted for the simple association of fiber population with average fiber weight and with average fiber diameter. The entire experiment by plants indicates that independent of all other fiber characters, average fiber length is negatively associated with average fiber weight per inch and that average fiber weight is positively associated with average fiber diameter. Selection of plants, or seeds, for higher population should lower the fiber length; selection of plants for longer fiber length should lower fiber weight; and selection for higher fiber weight should raise fiber diameter.

# RELATION OF DIET OF SWINE TO DEVELOPMENT OF LOCOMOTOR INCOORDINATION RESULTING FROM NERVE DEGENERATION<sup>1</sup>

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## INTRODUCTION

A troublesome locomotor disorder among pigs, manifested by incoordination in the use of the legs, abnormal posture, and lameness, which has been of common occurrence in the Bureau's herd at the United States Department of Agriculture, Beltsville Research Center, Beltsville, Md., was described in an earlier paper (1).<sup>2</sup> The observations there reported suggested both diet and breeding as possible causative factors, particularly among pigs confined in small pens and fed in record-of-performance tests. The role of breeding was suggested by the high incidence of lameness among inbred litters, and some significance was placed on the abnormally low free-choice intake of calcium- and phosphorus-rich components of the diet.

Although a lack of either vitamin A or vitamin D is recognized as causing characteristic types of lameness and paralysis, the disorder apparently persisted under a regimen considered adequate in these vitamins for growing and fattening pigs. It appeared that if the disease were of dietary origin, the cause was possibly an obscure derangement of calcium and phosphorus metabolism or suboptimum levels of an unknown vitamin or vitaminlike factor in the stock diet. An infection of swine erysipelas in the herd was also considered as a possible complicating factor since a small proportion of the growing pigs had swollen joints and other symptoms of this disease, which also occasionally resulted in lameness. However, no consistent gross lesions of the bones or joints, other than some possibly due to injury, were detected in the animals suffering from the type of incoordination under investigation.

Further study of the problem was accordingly undertaken along the following lines: Determination of the effects of mineral elements in the diet; search for easily available feeds for use as supplements or replacements in the stock diet which would completely prevent or cure the disease; and, in order to facilitate the identification of the deficiency factor, the formulation of an experimental diet or diets capable of producing the symptoms. Control of the lines of breeding of the animals used in experimental lots and of the incidence of erysipelas was coordinated with the dietary treatments. The results obtained are reported in this paper.

## REVIEW OF LITERATURE

A disease characterized by posterior paralysis and incoordination of movement was described by Wehrbein (7) of the Iowa State College

<sup>1</sup> Received for publication July 11, 1940.

<sup>2</sup> Italic numbers in parenthesis refer to Literature Cited, p. 316.

of Agriculture in 1916. Degeneration of the myelin sheath and of the brachial and sciatic nerves was observed. Efforts to demonstrate pathogenic organisms failed. In more recent years, Eveleth and Biester (2), also of the Iowa State College, have continued the work and have reviewed the reports of other investigators from the standpoint of the possible involvement of vitamin A and of the vitamin B complex in the nerve degeneration in various species. The complexity of the problem is evidenced by the conclusions of the Iowa investigators, which indicate that the incoordination and myelin degeneration in the nervous systems of swine are not necessarily associated in all cases and that neither vitamin A nor the vitamin B complex is responsible for the myelin degeneration.

In work on young pigs fed artificial diets, Wintrobe, Mitchell, and Kolb (7) observed poor growth, marked ataxia, and severe nerve and spinal-cord degeneration when the content of yeast was gradually reduced and thiamin and riboflavin substituted in the ration. In work at the Beltsville Research Center (6) on the vitamin B<sub>1</sub> requirement of young swine, nerve and spinal-cord degeneration have also been encountered in animals fed autoclaved diets with and without the addition of thiamin.

The observations of Hogan and coworkers (3) are also of interest because of their relation to sows and suckling pigs and because the development of the abnormal gait seemed to depend on sudden changes in the nutritive value of one or more constituents of the diet. These investigators reported serious losses during certain years in the pig crop, whereas in others the disease was mild in form or absent.

Hughes (4) has worked with purified diets in which the components of the vitamin B complex were varied. He observed marked lameness in pigs fed a diet low in riboflavin, although other groups on riboflavin-rich diets that were in turn low in thiamin, nicotinic acid, or other fractions of the B complex were not free of lame pigs.

Besides these reports, information in the possession of the Bureau, including communications from a number of workers at various State experiment stations, indicates that the disorder is of serious economic importance and is more prevalent in some years than in others.

#### EXPERIMENTAL PROCEDURE

Beginning with the spring farrow of 1937 and continuing through the spring farrow of 1939, groups of pigs were fed in concrete-floored pens under essentially the same conditions as those prevailing in record-of-performance tests except for dietary differences. Two experiments, one with spring and the other with fall pigs, were conducted each year. In the earlier experiments, the number of lots was restricted to six, and litters containing at least six pigs were utilized in order to provide litter mates in each lot. These litters were selected so far as possible from inbred stock which had shown a predisposition toward lameness, in an attempt to accentuate the effects of the various diets either in completely protecting against or in intensifying the symptoms. After 1937, this inbred stock became so depleted in the herd that it was not possible to continue this procedure. In the more recent experiments, it has been necessary also to depart somewhat from the use of complete litter-mate groups and

to select closely related pigs of similar age, sex, and weight. The pigs were generally placed on experiment at 10 to 12 weeks of age.

The diets were prepared from the general supply of feeds purchased for use in swine feeding at Beltsville. The stock diet consisted of No. 2 yellow corn, digester tankage of 60 percent protein content, linseed meal, alfalfa-leaf meal made from sun-cured hay, and a mineral mixture. This mineral mixture consisted of ground limestone, 50 percent; steamed bonemeal, 27.97 percent; common salt, 20 percent; iron oxide, 2 percent; potassium iodide, 0.02 percent; and copper sulfate, 0.01 percent. This mixture constituted 0.8 to 1.0 percent of the stock diet. The supplemental or replacement feeds were varied, being chosen because of known or supposed mineral, vitamin, or protein value and as contrasting feeds for replacement of the corn, tankage, linseed meal, or alfalfa-leaf meal.

The diets were adjusted, through changes in quantities of corn or other cereal and tankage, or its substitute, to furnish approximately 18 percent of protein to pigs between the weights of 40 and 100 pounds, 15 percent to those between the weights of 100 and 160 pounds, and 12 percent to those with weights of more than 160 pounds. Whenever linseed and alfalfa-leaf meals were used, they generally constituted 5 percent of the diet of the pigs in the lowest weight group, 4 percent of that of the middle group, and 3 percent of that of the highest weight group.

The diets fed were grouped in five series and are shown in table 1. In series A, particular attention was given to the effect of minerals on the production of lameness. As shown in the table, group 2 received a low mineral diet, and group 8 received a supplement of manganese chloride. Oats were used in group 6 in part because of their relatively high manganese content. Group 5 was given access to a small unpaved area of natural clay soil free of vegetation to determine whether the lacking element or elements in the diet could be obtained from the soil. Skim milk, green forage, and liver were fed in part for their mineral content but more in a search for a natural feed that would be consistently protective against the disease. The green forage consisted of both freshly cut green soybeans and alfalfa. It was estimated that the quantity fed corresponded to approximately 5 percent of the dry forage in the diet.

In series B, the use of corn-gluten meal in place of tankage and alfalfa-leaf and linseed meals was prompted by the deficiency of the first-named feed in riboflavin, as indicated by early assays for vitamin G, and the desire to determine the possible role of riboflavin in the prevention of the disease. Oats were used in group 2 because of the favorable results obtained in series A. Liver was used in the next group for its high riboflavin content and also for its richness in other members of the vitamin B complex. In one group molasses was also used for its richness in certain factors, including vitamin B<sub>6</sub>. Casein, washed with acidulated water and extracted with alcohol, was added in one group to improve the quality of protein mixture and as a control on the other diets to which natural feeds were added. Fortified cod-liver oil was added to the diets of series B, as well as to those of series C and D, in order to provide an adequate supply of vitamins A and D in all rations regardless of composition and treatment.

TABLE 1.—*Experimental set-up for the five series of tests*

## SERIES A—BASAL MIXTURE OF CORN, TANKAGE, ALFALFA-LEAF MEAL, AND LINSEED MEAL

Pig group No.	Trials	Pigs used in test	Proportion of basal mixture used	Diet supplement or treatment
	<i>Number</i>		<i>Percent</i>	
1	2	Spring and fall	99.2	Mineral mixture, 0.8 percent (stock control diet).
2	2	do	100	None.
3	2	do	100	5 pounds of skim milk per day.
4	3	Spring	100	1 pound of green forage per day. <sup>1</sup>
5	1	do	99.2	Same as group 1; pigs on earth runway.
6	2	Spring and fall	70	Oats, 30 percent.
7	1	Fall	97	Dried pork liver, 3 percent.
8	1	do	100	Manganese chloride, 72 p. p. m.

## SERIES B—BASAL MIXTURE OF CORN, CORN-GLUTEN MEAL, MINERAL MIXTURE, AND FORTIFIED COD-LIVER OIL

1	1	Spring	100	None.
2	1	do	70	Oats, 30 percent.
3	1	do	98	Dried pork liver, 2 percent.
4	1	do	95.5	Casein, 4.5 percent.
5	1	do	85	Molasses, 15 percent.

## SERIES C—BASAL MIXTURE OF TANKAGE, MINERAL MIXTURE, AND FORTIFIED COD-LIVER OIL

1	2	Spring and fall	13.1	Corn, 86.9 percent.
2	2	do	13.1	Barley, 86.9 percent.
3	2	do	13.1	Wheat, 86.9 percent.

## SERIES D—SIMPLE BASAL DIET OF CORN, TANKAGE, MINERAL MIXTURE, AND FORTIFIED COD-LIVER OIL OR STOCK CONTROL DIET OF ALFALFA-LEAF MEAL AND LINSEED MEAL ADDED TO SIMPLE BASAL DIET

1	3	2 spring and 1 fall	100	Simple, unheated (control).
2	3	do	100	Stock, unheated (control).
3	2	Spring and fall	100	Simple, heated.
4	1	Spring	100	Stock, heated.
5	1	Fall	90	Simple, heated, supplemented by—
6	1	do	97	Corn-gluten meal, 10 percent.
7	1	do	90	Dry liver, 3 percent.
8	1	do	92.3	Rice bran, 10 percent.
9	1	Spring	85.3	Dried whey, 7.7 percent.
10	1	do	85.3	Stock, heated, supplemented by—
11	1	do	99	Dried whey, 14.7 percent.
				Whey concentrate, 14.7 percent.
				Wheat-germ oil, 1 percent.

## SERIES E—CURATIVE TRIALS ON VARIOUS DIETS

1	1	Fall	100	Stock control, same as series D, No. 2.
2	1	do	100	Dried skim milk in place of tankage in series D, No. 2.
3	1	do	100	Animals fed with groups 1, 2, 6, 7, and 8 in series A.

<sup>1</sup> Alfalfa-leaf meal omitted from basal mixture.

In series C, comparison was made of corn, barley, and wheat, when constituting a high percentage of the diet, for the prevention of lameness.

In series D, the basal diet and a simplification of it, in which the linseed and alfalfa-leaf meals were omitted, were subjected to dry

heat at 115°–120° C. for 40 hours in the case of the former diet and 30 hours in the latter. Poultry-nutrition studies have shown that a factor or factors essential for the nutrition of the chick are destroyed by the heat treatment. The dermatitis produced by the feeding of a heated diet has been cured or prevented by the feeding of the vitamin B complex fraction designated as the filtrate fraction, and recent work has been reported (5) on the protective properties of pantothenic acid. Apparently, thiamin, riboflavin, vitamin B<sub>6</sub>, and possibly nicotinic acid are not inactivated or destroyed by the heat treatment. At the time that the first trials on the feeding of heated diets were planned (1938), it seemed evident that the factors just named were also not involved in the lameness derangement in pigs. In series D, as indicated in table 1, various supplements were used with the heated diets. The whey concentrate used in group 10 was prepared in the laboratories of the Bureau of Dairy Industry. In obtaining this product, whey from rennet-coagulated curd was evaporated and approximately two-thirds of the lactose allowed to crystallize out. Cod-liver oil was added to all diets at the time that new supplies of feed were placed in the self-feeders.

In series E, three groups of pigs are included that were segregated from the herd or from record-of-performance lots after definite signs of lameness had developed and were fed in an endeavor to determine whether cures or alleviation of symptoms could be obtained. One group received the stock diet, another a modified stock diet in which dried skim milk replaced the tankage, and the third consisted of a litter of five pigs that had been distributed among 5 lots in series A.

The pigs were kept on the test diets for periods of 12 to 24 weeks. As the experiments progressed, it was found that symptoms of lameness and incoordination were generally easily detected within a 12-week feeding period; consequently, the test period was shortened in later experiments. The mixed diets were self-fed and the consumption of each lot recorded. The pigs were weighed and examined weekly. A scoring system was set up by which to record the relative severity of the disease. Entire absence of symptoms was scored as 0; initial signs of lameness, as 1; more advanced lameness with tendency to weakness in pasterns and peculiar motion of rear legs, as 2 (fig. 1, A); definite incoordination shown by weaving motion and the throwing forward of the rear legs, also difficulty in rising and tendency to sit on the haunches, as 3; marked symptoms of those enumerated as 3, in which the animal had great difficulty in walking and frequently collapsed, as 4; and extreme paralysis, in which the animal was barely able to rise or to move about with the forelegs, as 5 (fig. 1, B).

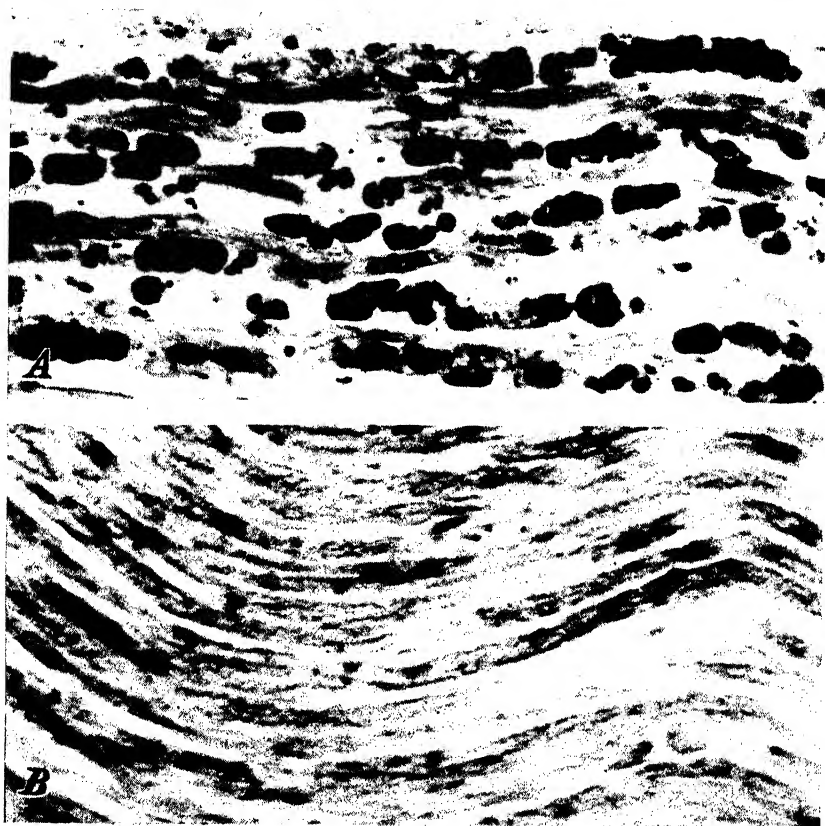
At the conclusion of the feeding period, the animals from most of the groups were slaughtered for laboratory studies. Samples taken included blood for corynebacteria tests, (made by the Pathological Division of the Bureau); also nerve and spinal cord tissue and the femur bones.

A histological study of the sciatic nerve and spinal cord of approximately 95 animals was made. Several peripheral nerves of the first few animals studied were sectioned for examination, but in the later work only the sciatic nerve and the spinal cord were taken for routine study since these tissues show the degeneration consistently when the other nerves are involved. The samples of nerve tissue were removed from the warm carcasses immediately after slaughter, and sections were



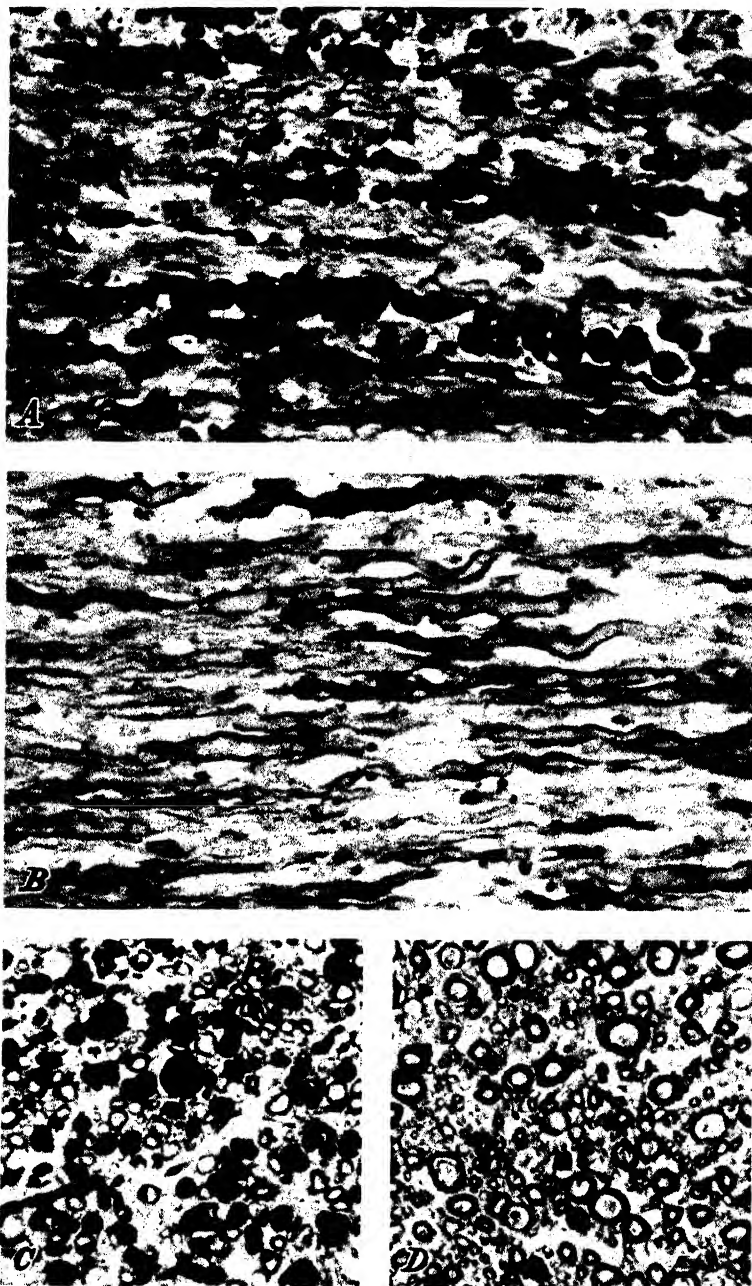
FIGURE 1.—A, Pig showing incoordination in the use of the hind legs. B, Pig with extreme paralysis, which developed while the animal was fed the heated diet No. 3 in series D.

prepared for study by the Marchi method. This procedure consists in placing the fresh tissues in a solution of potassium dichromate until the normal myelin is largely oxidized and then transferring to a water solution of osmium tetroxide and potassium dichromate to oxidize



*A*, Longitudinal section of the sciatic nerve from a typical animal with advanced locomotor incoordination showing massive demyelination of nerve fibers (black globular areas); *B*, longitudinal section of the sciatic nerve from a normal animal showing intact myelinated fibers.





*A*, Longitudinal section of a dorsal column of spinal cord from a partially paralyzed pig showing degeneration of myelinated tracts; *B*, longitudinal section of a dorsal column of the spinal cord from a normal animal showing normal medullated fibers; *C*, cross section of *A*; *D*, cross section of *B*.

further the degenerated myelin and ordinary adipose tissue. Oxidation of the products of the degenerated myelin sheaths results in a deposition in these areas of a lower oxide from reduced osmium tetroxide, which is black in color. The tissues were embedded in celloidin or paraffin, then cut and mounted in the usual way. The slides were either counterstained or examined directly for myelin degeneration, as shown by the globular black areas in plates 1 and 2. The absence, presence, and severity of myelin degeneration were scored on a numerical basis paralleling the method of scoring the live animals for lameness.

Supplementary to the hog-feeding experiments, a number of rat-feeding comparisons were made to determine the possible dietary deficiencies of the heated diets from the standpoint of rate of growth and feed utilization.

## EXPERIMENTAL RESULTS

### GROWTH OF PIGS AND INCIDENCE OF LAMENESS

Table 2 shows the results obtained with the pigs on the various series of diets. In series A, the animals in all eight groups grew at normal rates and remained on the diets until most of them weighed more than 200 pounds. As shown in the table, every group contained at least 1 affected animal. Of a total of 77 animals, 27 showed signs of lameness and incoordination. To a large extent, the cases were of a mild form. Perhaps the most striking result was the high incidence among the pigs fed green forage. The animals of this group (No. 4), like those of group 2, did not receive a mineral mixture, and the affected animals in these two groups showed a greater degree of lameness than those in any of the other groups.

Oats and dried liver were reasonably effective supplements. The former was fed as 30 percent of the diet, which is considered near the maximum for the most efficient utilization.

Most of the animals used in series A consisted of litters from the spring and fall farrows of 1937, many of which were selected from the inbred stock suspected as being predisposed toward the development of lameness. The data indicate that little was gained from the use of such stock to accentuate the effects of ordinary diets on the incidence and severity of incoordination. This finding has been confirmed by subsequent results with pigs representing a large number of lines of breeding and showing a varying incidence of incoordination from season to season.

In series B, the results obtained show rather definitely that the use of corn-gluten meal did not render the diets less effective in preventing lameness and incoordination. No affected pigs were found in either the control group or the group receiving oats, and only one mild case was recorded in each of the remaining groups. Growth on the control diet was poor. Although the rates of gain were highest with the feeding of liver and of casein, growth was still subnormal. Oats increased gains somewhat and molasses only slightly.

In series C, the three groups, regardless of the supplement fed, had similar rates of gain and final weights. The lowest incidence, as well as degree, of lameness was observed in the group receiving the barley supplement.

TABLE 2.—*Growth and incidence of lameness of groups of pigs on the five series of diets*

## SERIES A.—BASAL MIXTURE OF CORN, TANKAGE, ALFALFA-LEAF MEAL, AND LINSEED MEAL

Pig group No.	Pigs	Diet supplement or treatment	Average weight		Average daily gain	Feed per 100 pounds of gain	Lame animals	Average score for lameness
			Initial	Final				
	<i>Number</i>		<i>Pounds</i>	<i>Pounds</i>	<i>Pounds</i>	<i>Pounds</i>	<i>Number</i>	
1	11	Mineral mixture; control diet .....	43.6	204.6	1.50	370.9	3	0.67
2	11	None .....	48.6	214.1	1.72	356.4	5	.82
3	10	Skim milk .....	43.5	206.6	1.72	344.3	3	.50
4	18	Green forage .....	56.8	198.8	1.46	425.8	11	.98
5	5	Same as group 1; pigs on earth run-way .....	39.4	229.8	1.72	357.0	1	.10
6	10	Oats .....	46.8	219.7	1.68	381.7	1	.10
7	6	Liver .....	54.7	213.0	1.63	384.8	1	.10
8	6	Manganese .....	54.7	218.7	1.82	348.3	2	.25

## SERIES B.—BASAL MIXTURE OF CORN, CORN-GLUTEN MEAL, MINERAL MIXTURE, AND FORTIFIED COD-LIVER OIL

1	5	None .....	55.8	132.4	0.50	543.4	0	0.0
2	5	Oats .....	55.6	138.6	.75	467.3	0	.0
3	5	Liver .....	57.0	269.2	1.11	376.3	1	.2
4	5	Caseln .....	61.3	171.1	.93	438.1	1	.17
5	5	Molasses .....	56.8	152.8	.58	569.2	1	.20

## SERIES C.—BASAL MIXTURE OF TANKAGE, MINERAL MIXTURE, AND FORTIFIED COD-LIVER OIL

1	12	Corn .....	67.8	208.8	1.52	369.8	8	0.86
2	13	Barley .....	81.4	208.2	1.63	398.5	1	.04
3	13	Wheat .....	81.3	208.2	1.59	370.8	7	.42

## SERIES D.—SIMPLE BASAL DIET OF CORN, TANKAGE, MINERAL MIXTURE, AND FORTIFIED COD-LIVER OIL OR STOCK CONTROL DIET OF ALFALFA-LEAF MEAL AND LINSEED MEAL ADDED TO SIMPLE BASAL DIET

1	17	Simple, unheated (control) .....	63.8	211.8	1.54	363.7	11	0.90
2	17	Stock, unheated (control) .....	62.8	230.3	1.78	359.6	6	.41
3	12	Simple, heated .....	53.6	115.6	.52	451.7	12	2.59
4	6	Stock, heated .....	65.8	66.2	0	(1)	6	2.25
		Simple, heated, supplemented by—						
5	6	Corn-gluten meal, 10 percent .....	57.7	107.7	.48	402.7	3	1.00
6	6	Dry liver, 3 percent .....	56.8	207.1	1.52	336.1	2	.75
7	6	Rice bran, 10 percent .....	58.5	151.7	.93	397.5	2	.58
8	6	Dried whey, 7.7 percent .....	60.0	202.0	1.41	353.2	2	.67
		Stock, heated, supplemented by—						
9	6	Dried whey, 14.7 percent .....	77.0	182.5	1.21	408.4	1	.33
10	6	Whey concentrate, 14.7 percent .....	74.7	162.7	.99	488.8	3	.67
11	6	Wheat-germ oil, 1 percent .....	70.7	98.5	.31	810.2	6	1.75

## SERIES E.—CURATIVE TRIALS ON VARIOUS DIETS

Pig group No.	Pigs	Diet	Average weight		Average daily gain	Feed per 100 pounds gain	Lameness scores	
			Initial	Final			Beginning	End
	<i>Number</i>		<i>Pounds</i>	<i>Pounds</i>	<i>Pounds</i>	<i>Pounds</i>		
1	5	Same as series D, No. 2 .....	83.8	146.0	0.87	-----	2.6	2.5
2	5	Dried skim milk in place of tankage in series D, No. 2 .....	109.2	204.2	1.64	383.6	2.2	3.3
3	5	Animals fed with groups 1, 2, 6, 7, 8 in series A .....	70.8	215.0	1.85	380.2	2.0	1.3

<sup>1</sup> Daily consumption per pig approximately 1.95 pounds.

In series D, groups 1 and 2, the controls fed the unheated diets, grew normally but showed a seemingly high incidence of lameness, particularly group 1. The feeding of the unsupplemented heat-treated diets resulted in lameness and incoordination in varying stages in all 18 animals fed. Growth was also retarded in all pigs and in fact stopped in group 4. This group was on test for an average of 78 days. One animal died and others appeared near death as the experiment closed.

The supplements used stimulated growth in varying degrees. Dried pork liver, fed at a level of 3 percent, was very effective, and the commercial dried whey product, fed with the simple diet at a level of 7.7 percent, produced nearly as good growth. The whey concentrate, which was fed at a level approximating 210 cc. of fresh whey to 85 gm. of heated diet, was insufficient to prevent lameness completely although none of the three affected animals showed advanced incoordination. Of the five supplements used, wheat-germ oil was entirely ineffective in prevention of lameness, whereas the corn-gluten meal, liver, rice bran, and whey products were all moderately effective at the levels fed. The striking results obtained with the heated diet in the production of lameness, together with the protection afforded by these supplements when added to the heated diet, leave little doubt of the nutritional origin of the disease.

#### CURATIVE TESTS

In series E, the diets fed to the three groups of affected animals that had been segregated from their litter or lot mates failed to cure the lameness and incoordination. The results are shown in table 2. Although there was a minor improvement in the average scores of groups 1 and 3, the average of all three groups shows no improvement. The 5 pigs in group 2, which received dried skim milk, showed a decided increase in the severity of the disease. The failure of the curative tests may be due to the limited degree to which regeneration of nerve tissue can take place in the animal body.

#### RAT-FEEDING EXPERIMENTS

The rat-feeding experiments, the results of which are given in table 3, showed that the growth of rats was retarded by the heat treatment of the diets almost as much as it was in the pigs. The simple unheated diet, as used in pig series D, group 1, produced significantly poorer growth than the stock diet. In turn, the animals on the heated simple diet were retarded in growth and required more feed per unit of gain than those on the unheated diet.

The lack of a marked response from the addition of 5 percent of casein, extracted with water and dilute alcohol, indicates that quality of protein was not the limiting factor. Alfalfa-leaf meal fed at a 5-percent level, wheat germ at 10 percent, and rice bran at 5 percent appeared to be more effective supplements than the casein. When the daily allowance of rice bran was 1 gm. per day, or approximately 8 percent of the total food intake, the growth rate was nearly as great as when the unheated diet was fed, and was greater than for the wheat-germ supplement, of which the daily intake also averaged 1 gm., although constituting 10 percent of the diet. In the feeding of fractions of the rice bran, it was found that the filtrate fraction remaining

after the thiamin, riboflavin, and vitamin B<sub>6</sub> had presumably been removed by adsorption treatments was the most effective preparation of the series. Neither thiamin nor riboflavin preparations were of material benefit, thus supporting the findings with the rice-bran absorbate preparation that neither of these vitamins was a limiting factor in growth.

TABLE 3.—*Results of rat-feeding experiments on the heated pig diets of series D with various supplements*

Rat group No.	Rats used in experiments	Diet fed	Average gain in 12 weeks	Feed per gram of gain
	<i>Number</i>		<i>Grams</i>	<i>Grams</i>
1	8	Simple, unheated (control) .....	144.9	7.15
2	8	Stock, unheated (control) .....	185.1	6.55
3	14	Simple, heated .....	68.7	9.92
		Simple, heated, supplemented by --		
4	6	Casein, 5 percent .....	86.3	7.58
5	6	Alfalfa-leaf meal, 5 percent .....	100.2	7.37
6	6	Linseed meal, 5 percent .....	89.7	8.29
7	6	Wheat germ, 10 percent .....	96.2	8.49
8	4	Rice bran, 5 percent .....	98.0	7.96
9	4	Rice bran, 8 percent .....	138.3	7.22
10	4	Rice bran, absorbate .....	96.0	8.03
11	4	Rice bran, eluate .....	86.5	9.92
12	4	Rice bran, filtrate .....	135.5	7.75
13	6	Liver extract .....	164.7	6.33
14	6	Whey concentrate .....	145.8	6.45
15	6	Thiamin, 30 micrograms per week .....	80.3	8.71
16	6	Riboflavin, 105 micrograms per week .....	76.0	9.19

Liver, and to a less extent the whey concentrate, as prepared in the Bureau of Dairy Industry, were effective in promoting better growth than the unheated diet. The results as a whole appear to support the data presented by various workers that a factor of factors found in the so-called filtrate fraction obtained in the separation of the vitamin B complex are destroyed by prolonged dry-heat treatment.

#### HISTOLOGICAL STUDIES OF TISSUES OF PIGS

Histological examinations of tissues of pigs showed that a degeneration of the myelin sheaths in certain peripheral nerves and the spinal cord was probably a primary factor in the development of the locomotor symptoms evidenced in lameness and incoordination. As the work progressed, the similarity of the lesions to those reported by the Iowa investigators (2) and to those of Wintrobe and coworkers (8) became evident.

The nature of the degenerative changes in the myelin sheath of nerve fibers of the sciatic nerve and the spinal cord, as shown by the Marchi procedure, is illustrated in plates 1 and 2. Varying degrees of degeneration were found, sometimes involving only an occasional nerve fiber or tract and sometimes widespread areas.

The degenerative changes in the spinal cord were confined principally to the dorsal columns and dorsal nerve roots, although some demyelination was found in the ventral columns also. The dorsal columns of the cord are made up principally of ascending branches from the dorsal root fibers, and they convey posture, locomotor, and other sensations. An interruption of conduction in these tracts will lead to incoordination and finally a complete inability to walk, depending on the extent and location of the degeneration.

Table 4 presents a summary of the results of the histological examinations expressed numerically and of the outward symptoms of locomotor involvement as shown by the lameness score. The average scores for the outward symptoms are in general agreement with those for the nerve examinations. In most instances, the number of animals involved are not greatly different for the two scoring indices. Besides the animals included as having definite lesions, a number of others not included in the table had slight changes, from the normal structure, in the sciatic nerve and spinal cord, a condition which may or may not indicate early lesions. Even the unheated stock diet protected less than two-thirds of the animals. The finding of abnormalities in the pigs receiving green forage is confirmed by the histological studies on 10 animals. The data on this group indicate a condition intermediate between those on the unheated simple diet and those on the stock diet. The heated diet resulted in the greatest amount of spinal-cord degeneration, just as it did in general incoordination. The addition of dried whey in group 8 of series D prevented the extreme degeneration noted in group 3 of that series, and the higher level of whey used in group 9 was even more effective. Although the whey concentrate furnished considerable protection, it did not appear to be equal to the dried whey on an estimated liquid-whey basis. The addition of wheat-germ oil to the heated stock diet was of little if any avail in the prevention of lameness or of nerve degeneration. Rat tests showed the wheat-germ oil to be reasonably potent as a source of vitamin E, and it seems unlikely that the substances which confer vitamin E activity are involved in the nerve degeneration in question.

TABLE 4.—*Results of histological examinations on nerve tissues compared with data on incoordination*

Series	Group No.	Diet treatment or supplement	Pigs used	Incoordination data		Histological data on—			
				Pigs affected	Average score	Sciatic nerve		Spinal cord	
						Pigs with definite lesions	Average score	Pigs with definite lesions	Average score
			Number	Number		Number		Number	
A	4	Green forage	10	6	0.85	7	1.00	7	1.20
B	1	Control, corn-gluten meal	1	0	.00	1	.50	1	.50
B	2	Oat supplement	1	0	.00	0	.00	0	.00
B	3	Dried pork-liver supplement	1	0	.00	0	.00	0	.00
B	5	Molasses supplement	2	1	.50	1	.25	1	.25
C	6	Barley	6	1	.08	0		0	
C	3	Wheat	6	4	.50	3	.50	4	.67
D	1	Simple, unheated	15	12	1.00	11	1.43	14	1.47
D	2	Stock, unheated	16	6	.44	7	.41	7	.56
D	3	Simple, heated	12	12	2.59	12	3.17	12	3.42
D	4	Stock, heated	4	4	2.63	4	3.00	4	3.50
D	8	Simple, heated, supplemented with dried whey	6	2	.58	3	1.83	4	1.00
D	9	Dried whey	6	1	.33	2	.33	2	.50
D	10	Whey concentrate	6	2	.50	3	1.00	4	1.17
D	11	Wheat-germ oil	4	4	1.13	3	1.75	4	2.00

<sup>1</sup> Two additional animals died near the close of the experiment in advanced stages of paralysis, and no histological studies were made.

A comparison of the results for groups 2 and 3 of series C, which received barley and wheat respectively, with those for group 1 of series D, which received corn as the principal ingredient of the diet, showed that wheat proved little different from corn but that barley conferred a high degree of protection. Among the pigs examined, those fed barley and those fed corn-gluten meal were the only ones that showed no definite cases of nerve degeneration. A limited number of rats from the experiments already described were examined for myelin degeneration. Animals fed the heated diet with the cod-liver oil fed in separate dishes showed degeneration, and at least one of six animals showed evidences of incoordination.

### DISCUSSION

From the results thus far obtained, it seems reasonably certain that the locomotor symptoms and evidences of nerve and spinal-cord degeneration that have been found are similar to those reported by other investigators. However, Eveleth and Biester (2) concluded that the incoordination and myelin degeneration were caused by different etiological agents since severe incoordination was not always associated with advanced myelin degeneration. In some of their experiments, the feeding of diets deficient in vitamin A resulted in development of incoordination but only mild nerve degeneration.

Wintrobe and associates (8) describe demyelination in the peripheral nerves, the posterior root ganglia, and the posterior (or dorsal) columns of the spinal cord. Animals showing the degeneration had manifested "a peculiar slapping gait" and "were particularly awkward in turning." In the advanced stages, the animals became less active and sat with the hind legs sprawled in unnatural positions. The animals received an artificial diet from an early age, and the evidence pointed to a dietary deficiency of one or more components of the vitamin B complex other than thiamin or riboflavin.

In the present experiments, it was possible to control the production of the disease, within limitations, by the use of natural feedstuffs and thereby clarify some of the questions raised by the other investigators. Unlike the findings of the Iowa group (2), the results of the present work show a rather close association of incoordination with myelin degeneration. It seems apparent that the locomotor symptoms developed following the degeneration of fibers in the nervous system.

Thus far, repair of the degenerated fibers accompanied by disappearance of the symptoms of incoordination has not been observed. However, this result does not seem to be unusual, since a highly potent protective diet was not tested on the affected animals and regeneration of nerve tissue in the animal body is limited.

The finding that diets of the type commonly used in hog feeding, when subjected to heat treatment, will produce the disease has made it possible to study the problem in greater detail and with more certainty than formerly. There seems to be little doubt that the symptoms in live animals and myelin degeneration found histologically are similar in animals on heated diets and animals frequently affected on so-called normal diets. Heating of the diets undoubtedly destroyed or inactivated a nutritive factor or factors that are in some way related directly or indirectly to the nutrition of nerve fibers. Failure of animals to grow normally on the heated diets suggests that

more than one nutritive factor may be destroyed by heating. Observations on the growth of lame animals fed on unheated diets, including the stock or record-of-performance diet, as compared with normal animals under the same conditions, indicate that lame animals are frequently but not necessarily slower in rate of growth. Accordingly it is possible that the myelin-degeneration-preventive material is different from the growth factor destroyed by heating.

The results obtained on the heated diets have strengthened the opinion already expressed that breeding as a factor in predisposing the animals toward development of lameness is of much less concern than was at one time suspected. The absence of any serious cases of lameness in a number of litters from stock that had shown a high incidence in earlier work has contributed to this opinion.

Interference with normal bone development as a factor in the development of lameness has been eliminated by some work on the ash content of the femur bones.<sup>3</sup> However, the data on percentage of ash in the fat and moisture-free bone showed no relation to lameness or to diet. Another phase of the studies, namely, incidence of swine erysipelas, has been given some attention. Results of serological tests on blood serums, carried out in the laboratories of the Pathological Division of the Bureau, showed frequent positive cases of erysipelas, but there was no association of this disease with incoordination. However, lameness resulting from diseased joints due to erysipelas infections may be confused with the initial signs of incoordination due to dietary deficiency.

The identity of the lameness- and nerve-degeneration-preventive factor is still obscure. Evidently it is not present in large quantities in the usual swine feeds. Results suggest that barley and oats may be more effective preventives than corn and wheat. Liver, skim milk, whey, rice bran, and corn-gluten meal are among the feeds tested that possessed considerable value yet at the levels fed were not effective in all animals.

#### SUMMARY

The experimental work herein reported was carried on at the United States Department of Agriculture, Beltsville Research Center, Beltsville, Md., from the spring farrow of 1937 through the spring farrow of 1939. The pigs were generally placed on experiment at 10 to 12 weeks of age and were kept on test for 12 to 24 weeks. The disease, which frequently occurs among pigs confined in small pens with concrete floors, is characterized by incoordination in the use of the legs, abnormal posture, and lameness.

The diets used were prepared from the general supply of feeds purchased for use in swine feeding at Beltsville. The stock diet consisted of No. 2 yellow corn, digester tankage of 60-percent protein content, linseed meal, alfalfa-leaf meal made from sun-cured hay, and a mineral mixture. Various supplements or replacement feeds were used, being chosen because of known or supposed mineral, vitamin, or protein value or as contrasting feeds for those replaced in the stock diet.

Myelin degeneration of the nerves and spinal cord was demonstrated in the affected animals. The extent of degeneration was in general parallel with the severity of incoordination.

<sup>3</sup> Unpublished data.



When the normal diets were heated at 115°–120° C. for 30 to 40 hours, incoordination and myelin degeneration were generally produced in all animals. Heating greatly increased the incidence and severity of the disease. The incidence of the disease was high on stock diets commonly used for growing and fattening hogs.

Supplemental or replacement feeds incorporated into the basic diets, including the heated diets, gave favorable results in some cases although complete protection was infrequent. Of the various feed materials investigated, liver, concentrated milk products, barley, and oats afforded the greatest protection.

Animals that did not receive a mineral supplement in the diet tended to show a greater degree of lameness than those receiving such a supplement.

The results indicate a deficiency disease of nutritional origin. The dietary factor appears to be present in variable or insufficient quantities in many of the feeds commonly used in hog feeding and is destroyed or inactivated by dry-heat treatment.

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## EFFECT OF COOL STORAGE OF EASTER LILY BULBS ON SUBSEQUENT FORCING PERFORMANCE<sup>1</sup>

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### INTRODUCTION

Easter lily bulbs produced in the Southern States usually are ready to dig before the florist is ready to receive them for forcing. Some storage period is therefore necessary. The present study conducted at the United States Horticultural Station, Beltsville, Md., was begun in the fall of 1935 with the purpose of finding a storage practice that would maintain the forcing quality of the bulbs and reduce to a minimum the occurrence of rot, shriveling, and premature sprouting. Short exposures to cool temperature kept the bulbs in good condition and showed a marked effect in accelerating bloom. This latter effect was examined in further detail, and the present paper summarizes 3 years' trials showing that specific preplanting storage treatments are a convenient means of controlling the time of flowering in Easter lilies.

### MATERIAL AND METHODS

The horticultural varieties of *Lilium longiflorum* Thunb. included in these studies and the sources of the experimental material were Creole from Louisiana, Croft from Oregon, Erabu and Giganteum from Japan, and Harrisii from Bermuda. The name "Croft" has been applied for convenience to a commercial type grown in Oregon without varietal designation and purchased direct from growers each year. One of the 1936-37 stocks of Creole and the 1937-38 stock of Harrisii were also bought direct from growers. All others were purchased through dealers. Attempts were made to obtain each stock promptly after digging to avoid the complication of undesired storage practices before the bulbs were received for the experiments. In general these attempts were not successful, and the resulting discrepancies will be discussed herein.

Bulbs 7 to 9 inches in circumference were specified in all purchases. The mean circumference was determined from 50 random bulbs of each variety and ranged from 7.20 to 8.19 inches, with the exception of one Creole lot (C<sub>1</sub> 37 in table 2) in which the circumference averaged only 5.57 inches. The last-named lot was supplied after standard sizes had been sold. The *Lilium longiflorum* seedlings included in one trial were random samples of open-pollinated progenies of the Croft variety. These had been grown in the greenhouse approximately 1 year from seed when the trials were begun.

<sup>1</sup> Received for publication November 25, 1940.

The number of bulbs per unit trial was 25, but if one or more failed to grow or to flower, the means were computed for the number flowering. This was considered sound practice, since the factors responsible for failure to grow or to bloom were apparently independent of the factors under test. Two packing materials, moistened peat moss and dry sand (or soil), were used. Throughout the 1935-36 and 1936-37 studies the individual test lots were repacked for storage tests in peat moss which had been thoroughly wet and from which the excess water was pressed out with the hands. In 1937-38 this moist-peat pack was compared with the original dry sand (Bermuda) or sandy loam (Louisiana) in which the bulbs were shipped.

Storage facilities were provided by the storage and transportation project of this Division at the Arlington Experiment Farm, Arlington, Va. Temperatures were controlled within a range of approximately  $\pm 1^\circ$  F. from the values stated, and relative humidity was maintained at a level of approximately 80 percent. Bulbs subjected to coldframe treatment were potted as for greenhouse planting and plunged in an open unheated frame, where they were exposed to the outdoor temperatures of Beltsville, Md., for the stated intervals. At the close of these intervals the pots were moved to the greenhouse.

In the greenhouse the bulbs were grown singly in 6-inch clay pots in a composted soil with which a liberal amount of bonemeal had been well mixed. The soil for each year's series of trials was prepared and mixed in advance. All trials were conducted in the same section of a greenhouse, the temperature of which was manually controlled, as far as practicable, to  $65^\circ$  F. in the daytime and  $60^\circ$  at night. These temperatures, however, were often far exceeded during the summer months.

#### 1935-36 EXPERIMENT

The data for 3 varieties included in the first year's trials (1935-36) are shown in table 1. Since these varieties were received at different dates, the storage exposures and corresponding control plantings were made independently for each variety. It is apparent from table 1 that cool storage accelerates blooming, the difference in number of days to bloom between stored and control lots always proving highly significant. The height of flowers above the soil line also shows significant decreases in the stored lots as compared with the nonstored lots in the Croft variety. The number of flowers per bulb is decreased in stored lots of Croft and Erabu but not in Creole. The actual date of bloom is shifted less strikingly by storage treatment than is the number of days to bloom, since the stored lots were planted (and the coldframe lots moved to the greenhouse) 37 to 41 days later than the control lots. Data in table 1 from unselected seedlings exposed for two different intervals in the coldframe and those without such exposure to a cool period show similar trends.

Although the general trends toward earlier blooming, shorter stems, and fewer flowers were similar in the three varieties and in one group of seedlings tested, these responses were most striking in the Croft variety. In actual flowering dates the stored lots of Croft were far in advance of the controls, while the stored lots of Erabu bloomed slightly later but within 6 days of the controls. Accordingly the 1936-37 trials were planned to compare the response of different Easter lily varieties to similar storage treatments.

TABLE 1.—Effect of storage treatment on time of blooming and quality of flowers of 4 varieties of Easter Lily in 1935-36

Variety and source	Circumference of bulbs on arrival <sup>1</sup>	Preplanting treatment	Date planted in greenhouse	Date of blooming	Days to blooming <sup>2</sup>	Height <sup>3</sup>	Flowers per bulb <sup>4</sup>
	Inches		1935	1936	Number	Inches	Number
Croft, from Oregon.	7.92±0.07	None	Oct. 8	June 2	238.3±1.98	20.0±1.00	7.4±0.37
		Coldframe 41 days	Nov. 15	May 21	188.8±1.26	13.6±.35	6.4±.21
		Stored at 50° F. 41 days.	do	Apr. 26	162.9±.82	11.6±.31	3.4±.14
		Stored at 36° F. 41 days.	do	Apr. 21	158.2±.99	13.3±.33	3.4±.13
Creole, from Louisiana.	7.49±.06	None	Oct. 25	Apr. 26	184.4±1.10	26.3±.38	4.3±.16
		Coldframe 40 days	Dec. 4	Apr. 17	135.6±.56	24.0±.44	4.2±.16
		Stored at 50° F. 40 days.	do	Apr. 21	139.0±1.08	23.8±.40	3.7±.24
		Stored at 36° F. 40 days.	do	Apr. 24	142.6±.74	23.0±.47	4.2±.16
Erabu, from Japan.	7.87±.10	None	Nov. 12	do	164.5±1.44	19.6±.80	7.2±.42
		Coldframe 37 days	Dec. 19	Apr. 25	128.0±1.01	17.2±.81	5.6±.40
		Stored at 50° F. 37 days.	do	Apr. 28	131.8±1.16	20.7±.67	4.7±.43
		Stored at 36° F. 37 days.	do	Apr. 30	133.2±.94	18.8±.82	5.3±.26
<i>L. longiorum</i> (seedling).		None	Jan. 8 <sup>3</sup>	June 3	144.7±3.43	23.6±1.35	5.4±.29
			1936				
		Coldframe 81 days	Jan. 10	May 5	116.6±.74	17.8±.56	6.0±.44
		Coldframe 182 days	Apr. 20	June 13	54.1±.70	15.6±.49	2.6±.20

<sup>1</sup> Mean and standard error.<sup>2</sup> From date of planting in greenhouse.<sup>3</sup> Date on which seed was planted.<sup>4</sup> Computed as days after Jan. 10, 1936.

## 1936-37 EXPERIMENT

In the experiment of 1936-37 the varieties Giganteum and Harrisii were added to those previously tested. Two lots of Erabu, designated by the dealer as "Black Stem Regular" (E<sub>2</sub>37 in table 2) and "Improved Early Flowering" (E<sub>1</sub>37 in table 2), were included. The dealer did not state whether the early type was genetically or physiologically early, but the results obtained by the writer suggest that its difference from the Black Stem Regular is attributable to handling rather than to selection. Two stocks of Creole were used; one of regular commercial size obtained from a dealer after considerable delay, and one of smaller bulbs obtained direct from the producers. The Croft variety was delayed in arrival because of forest fires in Oregon.

Since it was planned to start storage trials on all varieties simultaneously to minimize differences in the conditions encountered during forcing, the early arrivals were held in their original packing until the later arrivals were received. The prolonged delay of Croft and Creole (C<sub>2</sub>37) introduced an error that had not been anticipated. The early-arriving varieties stood for 5 to 7 weeks in the original cases in a room at 50° F., which constituted a cool storage treatment in the controls. The effect of this storage is shown in differences that appear between lots 1A, planted on arrival, and lots 1, planted November 2, after all lots (except Croft) were on hand. The duration of this unplanned storage interval may be computed from the differences between planting dates of lots 1A and 1 in table 2. Where no lot 1A appears, the variety was received just in time for the tests proper.

TABLE 2.—Effect of storage treatment on time of blooming and quality of flowers in 1936-37

CREOLE C<sub>37</sub> (MEAN CIRCUMFERENCE OF BULBS, 5.57±0.5 INCHES)

Lot No.	Storage treatment		Date planted in greenhouse	Average date of blooming	Days to blooming <sup>1</sup>	Days to emergence <sup>1</sup>	Height <sup>2</sup>	Flowers per plant <sup>2</sup>	Size of flower <sup>2</sup> (length by diameter)
	Temperature	Interval							
1A	° F. (3)	Weeks	1936	1937			Inches	Number	Index number
1			Sept. 12	Apr. 14	214.0±0.70	69.1±0.80	27.1±0.57	3.5±0.21	28.5±0.47
2			Nov. 2	Apr. 10	158.7±.58	35.7±.91	31.1±.53	3.0±.18	29.1±.38
3	50	5	Dec. 7	Apr. 12	125.8±.90	18.1±1.36	30.7±.89	2.7±.53	29.1±.60
4	40	5	do	Apr. 16	129.8±.68	28.7±.90	29.4±.49	3.1±.10	28.8±.41
9	32	5	do	Apr. 22	135.6±.82	34.2±.73	27.8±.47	3.4±.16	29.6±.51
	(5)	5	do	Apr. 18	131.9±.70	26.2±.75	27.4±.64	3.4±.15	29.8±.59
6	50	10	1937						
7	40	10	Jan. 11	Apr. 22	101.0±.97	.6±.56	24.4±.49	1.3±.11	30.1±.69
10	32	17	do	May 6	115.0±1.36	28.3±1.68	18.3±.74	2.4±.54	27.6±.66
			Mar. 5	May 23	78.6±1.05	17.9±1.00	13.9±.55	2.4±.39	25.1±.46

CREOLE C<sub>37</sub> (MEAN CIRCUMFERENCE OF BULBS, 7.55±0.15 INCHES)

1	(4)		1936						
2	50	5	Nov. 2	Mar. 16	134.4±1.62	22.4±1.02	32.3±1.30	3.7±0.56	28.9±0.33
3	40	5	Dec. 7	Apr. 6	120.2±1.13	20.0±1.24	31.4±.90	2.9±.34	27.9±.47
4	32	5	do	Apr. 14	128.0±1.31	28.7±1.41	30.9±.82	4.0±.43	28.5±.56
9	(4)	5	do	Apr. 16	130.5±1.25	29.0±.81	33.1±.71	4.0±.39	28.1±.72
			do	Apr. 10	124.2±1.60	19.5±1.23	29.9±1.04	4.4±.48	28.1±1.22
6	50	10	1937						
7	40	10	Jan. 11	Apr. 27	105.6±1.52	5.6±1.53	22.7±.87	1.7±.20	29.5±.66
			do	May 9	117.8±1.67	28.1±1.79	18.1±1.01	3.2±.39	27.5±.68

## CROFT CT 37 (MEAN CIRCUMFERENCE OF BULBS, 7.28±0.08 INCHES)

1	(4)		1936						
2	50	5	Nov. 5	Apr. 22	167.9±4.18	35.7±1.02	14.0±0.65	5.8±0.35	29.4±0.89
3	40	5	Dec. 7	Apr. 29	142.6±2.37	25.4±1.35	13.1±.31	5.8±.20	28.6±.89
4	32	5	do	May 2	145.5±2.29	35.8±1.73	13.9±.65	4.6±.28	30.9±.72
9	(4)	5	do	Apr. 30	143.9±1.84	37.0±1.26	11.2±.51	5.0±.33	23.2±.68
			do	May 2	145.8±1.71	33.6±1.11	11.3±.53	4.5±.29	29.8±.54
6	50	10	1937						
7	40	10	Jan. 11	May 12	121.4±1.75	15.6±2.62	10.9±.38	3.2±.28	30.4±.72
10	32	17	do	May 13	122.3±1.21	30.2±.92	10.3±.21	4.7±.35	28.2±1.93
			Mar. 5	May 24	79.8±.85	17.4±.47	12.5±.36	5.4±.58	28.0±.53

EARLY ERABU E<sub>37</sub> (MEAN CIRCUMFERENCE OF BULBS, 8.19±0.11 INCHES)

1A	(3)		1936						
1	(4)		Sept. 26	Feb. 24	150.7±6.39	47.2±3.55	20.5±1.12	3.4±0.23	23.5±1.08
2	50	5	Nov. 2	Mar. 12	129.5±2.44	28.4±.84	25.9±.87	4.5±.33	24.5±.67
3	40	5	Dec. 7	Mar. 30	113.4±1.12	12.8±.88	26.5±1.15	4.2±.27	23.0±.50
4	32	5	do	Apr. 8	122.1±2.10	26.5±1.16	20.0±.85	5.8±.72	23.2±.90
9	(3)	5	do	Apr. 11	124.8±1.67	30.5±1.09	22.8±1.07	4.0±.34	23.8±1.76
			do	Apr. 4	118.5±1.56	19.9±.77	23.7±1.32	4.7±.35	24.2±.94
6	50	10	1937						
7	40	10	Jan. 11	Apr. 21	99.6±1.32	9.7±2.46	14.2±.63	2.5±.23	22.2±.79
			do	Apr. 30	109.2±1.67	18.8±1.48	15.7±.75	2.4±.13	22.3±.82

ERABU E<sub>37</sub> (MEAN CIRCUMFERENCE OF BULBS, 7.64±0.10 INCHES)

1A	(3)		1936						
1	(4)		Sept. 26	Mar. 14	169.0±2.47	45.3±1.80	20.6±0.92	3.7±0.41	21.6±0.63
2	50	5	Nov. 2	Apr. 1	149.5±1.75	34.2±1.65	28.0±.94	5.4±.41	23.5±.75
3	40	5	Dec. 7	Apr. 12	125.7±1.48	16.1±.79	28.1±.82	4.6±.34	24.2±.74
4	32	5	do	Apr. 18	132.1±1.20	26.8±.87	25.1±.84	4.4±.26	22.9±.60
9	(3)	5	do	Apr. 19	133.3±1.48	30.6±.78	21.6±.81	5.7±.33	22.6±.56
			do	Apr. 24	138.5±1.58	27.4±.87	23.3±.99	5.1±.33	24.2±.61
6	50	10	1937						
7	40	10	Jan. 11	May 5	114.4±2.23	10.4±2.41	18.1±.87	2.9±.25	22.3±1.11
10	32	17	do	May 9	117.9±1.50	25.2±1.39	16.2±.85	3.0±.78	22.6±.53
			Mar. 5	June 1	88.3±1.67	25.5±1.55	19.8±.70	3.1±.53	22.9±.91

Footnotes at end of table.

TABLE 2.—Effect of storage treatment on time of blooming and quality of flowers in 1936–37—Continued

GIGANTEUM (MEAN CIRCUMFERENCE OF BULBS,  $7.20 \pm 0.07$  inches)

Lot No.	Storage treatment		Date planted in greenhouse	Average date of blooming	Days to blooming	Days to emergence <sup>1,2</sup>	Height	Flowers per plant <sup>3</sup>	Size of flower (length by diameter)
	Temperature	Interval							
	° F.	Weeks	1936	1937			Inches	Number	Index number
1A ..	(3)	.....	Oct. 20	May 5	197.0 $\pm$ 1.00	33.5 $\pm$ 0.73	15.2 $\pm$ 0.55	5.5 $\pm$ 0.21	24.4 $\pm$ 0.43
1 .....	(4)	.....	Nov. 2	May 4	182.6 $\pm$ .96	37.1 $\pm$ 1.09	14.0 $\pm$ .56	5.8 $\pm$ .43	24.1 $\pm$ .84
2 .....	50	5	Dec. 7	do	148.1 $\pm$ 1.32	20.3 $\pm$ 1.11	13.7 $\pm$ .57	4.7 $\pm$ .34	24.4 $\pm$ .56
3 .....	40	5	do	May 9	153.0 $\pm$ 1.28	33.1 $\pm$ 1.20	12.1 $\pm$ .48	4.4 $\pm$ .23	24.2 $\pm$ .41
4 .....	32	5	do	May 11	155.2 $\pm$ 1.44	36.4 $\pm$ 1.44	9.0 $\pm$ .44	5.0 $\pm$ .32	22.2 $\pm$ .66
9 .....	(5)	5	do	May 4	148.2 $\pm$ .99	51.3 $\pm$ 1.77	10.1 $\pm$ .41	5.0 $\pm$ .29	24.2 $\pm$ .61
6 .....	50	10	Jan. 11	May 14	122.8 $\pm$ 1.40	11.0 $\pm$ 3.16	9.1 $\pm$ .46	2.6 $\pm$ .22	23.5 $\pm$ .98
7 .....	40	10	do	do	122.8 $\pm$ .96	29.7 $\pm$ 2.74	9.3 $\pm$ .35	3.1 $\pm$ .19	24.5 $\pm$ .48
10 .....	32	17	Mar. 5	June 2	89.2 $\pm$ 2.13	24.6 $\pm$ 1.34	13.2 $\pm$ .96	2.7 $\pm$ .22	24.3 $\pm$ 2.34

HARRISH (MEAN CIRCUMFERENCE OF BULBS,  $7.97 \pm 0.10$  inches)

1A ..	(4)	.....	1936	May 1	217.3 $\pm$ 2.33	86.0 $\pm$ 2.21	20.5 $\pm$ 0.90	2.7 $\pm$ 0.30	30.7 $\pm$ 2.18
1 .....	(4)	.....	Sept. 26	May 3	181.8 $\pm$ 3.23	55.2 $\pm$ 3.72	21.6 $\pm$ .78	3.2 $\pm$ .25	32.0 $\pm$ 1.46
2 .....	50	5	Nov. 2	May 16	160.2 $\pm$ 2.80	40.7 $\pm$ 2.54	18.5 $\pm$ .90	2.7 $\pm$ .24	31.6 $\pm$ .85
3 .....	40	5	Dec. 7	May 14	157.6 $\pm$ 3.04	39.9 $\pm$ 1.07	18.6 $\pm$ .82	2.9 $\pm$ .25	34.0 $\pm$ .58
6 .....	50	10	1937	May 24	133.4 $\pm$ 3.56	25.6 $\pm$ 5.57	16.8 $\pm$ .84	2.5 $\pm$ .23	32.5 $\pm$ .85
7 .....	40	10	Jan. 11	May 28	137.2 $\pm$ 2.51	33.2 $\pm$ 2.51	17.5 $\pm$ .89	2.9 $\pm$ .16	33.2 $\pm$ .90

<sup>1</sup> From date of planting in greenhouse.<sup>2</sup> Mean and standard error.<sup>3</sup> Bulbs planted in greenhouse on arrival.<sup>4</sup> Bulbs planted in greenhouse Nov. 2 (except Croft).<sup>5</sup> Unheated coldframe.

The storage temperatures and intervals and the forcing data are detailed in table 2. An index of the size of flower, that is, the product of the length by the diameter of the flower when approximately prime, is also presented in table 2. This index is believed to be a more reliable measure than length or diameter alone, since these measures change as the flower opens and fades but tend to equalize each other in the index. The difference between any two means is treated as significant (5-percent level) when found to be twice its standard error.

Bulbs kept in cool storage bloomed in a shorter time from date of planting than the controls in all cases, the differences proving highly significant with rare exceptions (C<sub>2</sub>37, lot 4). However, the actual dates of blooming were never significantly earlier than those of the untreated lots. The maximum effects of storage treatments appear in Creole C<sub>1</sub>37, the stock shipped to Beltsville direct from Louisiana. Significant shortening of the number of days required for flowering of stored lots appeared in all the varieties tested, indicating that the accelerating effect of low temperature is not peculiar to any one type of Easter lily. The degree of acceleration of flowering brought about by cool storage again varied among varieties, but the variety most influenced by treatment in 1935–36 (Croft) was relatively unresponsive in 1936–37. Moreover, the control lots of the two stocks of Creole included in the 1936–37 trials showed a striking difference in



blooming time. This is most probably attributable to handling before these two stocks reached Beltsville, since all commercial Creoles are grown and harvested under rather closely comparable conditions. The effect of cool storage is cumulative, and Creole C<sub>2</sub>37 apparently had undergone some cooling before it was received. Additional cool storage applied to a commercial lot already exposed to some similar treatment still shows a significant shortening of the time required for blooming, but the full effect of the treatment is obscured by the failure of the control lot to show the expected delay in flowering. It seems futile, therefore, to attempt to determine varietal differences in response to these treatments by using material so diverse in origin and prior handling. Genetic differences in capacity to respond presumably occur, but they are hopelessly confounded with physiological differences due to different sites of production and methods of handling.

Storage at 50° F. was in general more effective in inducing earliness than either 40° or 32° for comparable intervals, but the differences are not consistently significant. This point is suggested in the data in table 2, but will be shown more clearly later. Storage at 50° or 40° for 10 weeks induced more prompt blooming for all lots than 5 weeks' storage at the same temperature. The lots stored 17 weeks at 32° all bloomed much more promptly than comparable lots stored for shorter periods. These lots (No. 10) of course received the additional stimulus of higher greenhouse temperature, which rises beyond control in May at Beltsville, Md.

The time required for the plant to emerge from the soil shows great irregularity. Some stocks (Creole C<sub>1</sub>37, Early Erabu, Erabu, and Harrisii) emerged more promptly after storage treatment. This was a material advantage in Harrisii, which was slow to emerge when not placed in cool storage. Apparently cool storage reduces the importance of the common practice of starting bulbs at a low temperature, which is raised after growth has started. Storage at 50° F. for 10 weeks was followed by prompt emergence of all varieties except Harrisii. Both shoot and root growth occurred at this temperature, but the resulting plants were not salable because the etiolated shoots developed in storage never expanded to normal size. Moist storage at 50° should not be practiced except for short intervals, such as 4 to 6 weeks, as a means of inducing extreme earliness.

That cool storage has a tendency to induce flowering while the plants are short is shown in table 2 by significant shortness of plants in all lots stored 10 weeks or more at 32°, 40°, or 50° F. The 5-week storage intervals in general showed no significant reduction in height. There was also a tendency for cool-stored bulbs to produce fewer flowers than the controls. This point is illustrated in table 2 by the number of flowers in lots stored 10 weeks or more, which is usually, but not always, significantly lower than the number for the corresponding control lot. The effects of cool storage on both height and number of flowers are better illustrated in data to be discussed in a later section (p. 328).

The interval between the first and last flowers on a single plant, which was visualized as an index of duration of attractiveness of a lily as a pot plant, was also measured. The data have not proved very helpful, however, so they are not included. They appear to be dependent on the number of flowers per plant as well as on the lasting

qualities of individual flowers and on other factors. Low values obtained for the lots stored for 10 weeks at 50° F. are largely a reflection of a reduced number of flowers in these lots.

The mean size of flower is a very stable characteristic of the variety as compared with other characteristics shown in table 2. Very few differences in this column show evidence of significance.

### 1937-38 EXPERIMENT

#### CREOLE VARIETY

Since comparisons of varietal response had not proved enlightening, the 1937-38 experiments were confined chiefly to the Creole variety, which could be obtained shortly after digging, with the addition of a simple interval test with *Harrisii* designed to extend the bloom of this variety over 2 or 3 months. Both varieties were received on August 26 direct from producing areas with no exposure to cool storage before shipment. Storage trials were set up immediately on receipt of the stocks.

The experiments were intended to provide comparisons between moistened-peat and dry-soil storage and also to afford an opportunity to examine further the effect of the length of the storage interval. The 40° and 50° F. storage trials were not carried beyond 15 weeks' duration, since the 1936-37 results had shown that these temperatures, if long continued, were likely to produce unfavorable effects. The 32° storage was continued up to 60 weeks to determine the tolerance of the Creole variety to this temperature, which in previous trials had appeared least deleterious in its effects on forcing quality. It was hoped that the bloom of the Creole variety could be extended throughout an entire calendar year by suitable storage manipulation. This result was indeed attained, but the average forcing quality of the 7- to 9-inch bulbs was poor after 60 weeks' storage at 32°.

The 1937-38 trials with the Creole variety are summarized in table 3. It will be noted at once that the number of days required to bloom is very greatly reduced by all storage practices included in these trials. In fact, all 5 weeks' treatments, all 10 weeks' treatments, and 2 of the 15 weeks' treatments were significantly earlier in actual date of blooming than the control lot, which flowered during Easter week. These differences are readily visualized by reference to figure 1. These clear-cut differences, which contrast with the less satisfactory results of the previous year, are attributed to the fact that this Creole stock was received before it had been subjected to previous storage.

The earliest bloom was produced by lot 4, which was stored 5 weeks at 50° F. in moist peat. The flowers were of satisfactory size and quality but sharply reduced in number (3.6 per plant), and the plants were significantly shorter than the controls; however, they were very acceptable Easter lilies for February. Lot 7, stored dry at 50° for 5 weeks, bloomed about 14 days later, with no significant improvement in the number of flowers. Longer storage at 50° (10 weeks and 15 weeks, dry) reduced the number of flowers to about two per plant. It seems clear that long storage at 50° is undesirable, and that this temperature is useful only in inducing extreme earliness of bloom.

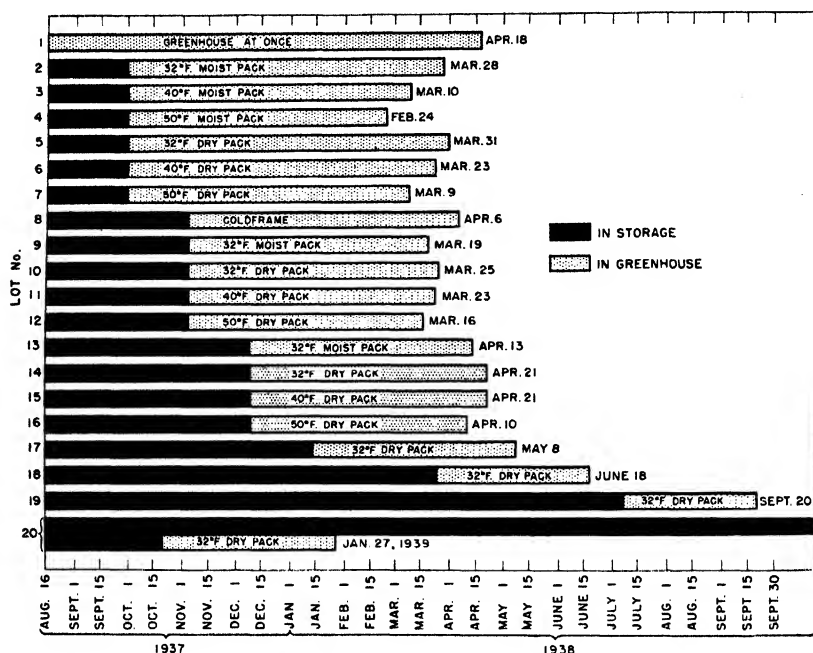


FIGURE 1.—Chart of 20 storage trials with Creole Easter lilies in 1937-38, showing relative length of time in storage and in the greenhouse and mean flowering data.

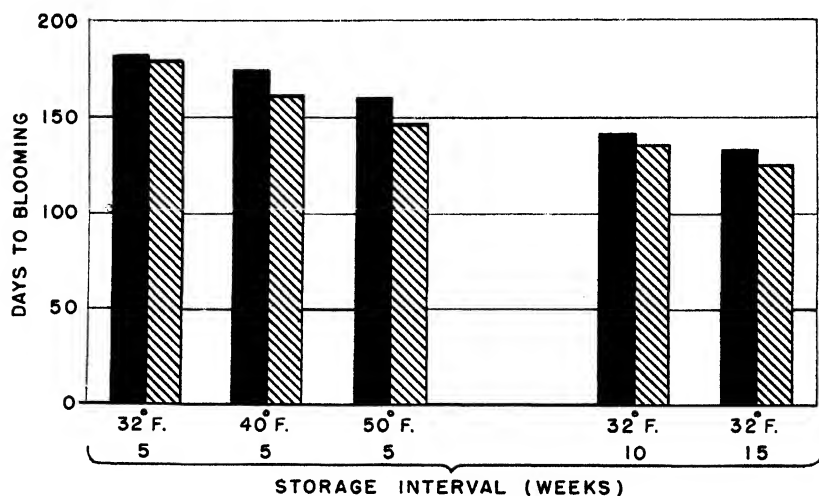


FIGURE 2.—Charts showing comparative effects of storage in dry pack and in moist pack (shaded) on days to blooming in Creole Easter lilies in the 1937-38 trials.

TABLE 3.—Effect of storage treatment on time of blooming and quality of flowers of the varieties *Creole* and *Harrisii* in 1937-38  
CREOLE C<sub>38</sub> (MEAN CIRCUMFERENCE OF BULBS, 7.64±0.05 INCHES)

Lot No.	Storage treatment		Date planted in greenhouse	Average date of blooming	Days to blooming <sup>2,3</sup>	Days to emergence <sup>2,3</sup>	Height <sup>3</sup>	Flowers per plant <sup>3</sup>	Leaves per plant <sup>3</sup>	Size of flower <sup>3</sup> (length by diameter)
	Temperature	Interval								
	° F.	Weeks								
1 <sup>4</sup>	32	5	1937 Aug. 26	1938 Apr. 8	Number 233.3±0.65	Number 42.0±0.57	Inches 34.6±0.83	Number 8.5±0.19	Number 162.7±1.19	Index 23.5±0.54
2	32	5	Sept. 30	Apr. 28	179.9±0.78	21.9±0.37	21.5±0.55	3.1±0.16	102.5±1.31	23.5±0.54
3	40	5	do	Mar. 28	166.9±0.78	23.0±0.45	20.1±0.45	3.2±0.17	84.9±1.21	23.5±0.54
4	40	5	do	Mar. 24	166.8±0.91	23.0±0.45	20.1±0.45	3.2±0.17	84.9±1.21	23.5±0.54
5	32	5	do	Mar. 31	182.0±0.71	33.9±0.75	22.6±0.74	4.2±0.10	106.5±1.12	25.0±0.59
6	32	5	do	Mar. 23	174.2±0.72	33.5±0.65	20.0±0.57	4.2±0.10	106.5±1.12	25.0±0.59
7	50	5	do	Mar. 9	160.4±0.67	26.6±0.70	19.2±0.44	3.6±0.19	73.9±1.63	25.8±0.49
8	(1)	10	Nov. 4	Apr. 6	153.2±1.06	25.4±0.49	19.3±0.60	6.0±0.18	99.6±1.58	25.3±0.48
9	32	10	do	Mar. 19	135.2±1.66	30.1±0.60	24.1±0.56	4.8±0.14	70.9±1.51	26.5±0.48
10	32	10	do	Mar. 25	141.4±0.65	31.5±0.98	26.0±0.65	4.5±0.17	63.6±0.89	26.4±0.46
11	40	10	do	Mar. 23	139.1±0.95	29.4±0.50	24.8±0.44	4.2±0.10	52.4±0.72	26.9±0.45
12	50	10	do	Mar. 16	131.7±1.05	29.4±0.50	18.9±0.57	2.2±0.22	46.9±0.72	27.0±0.67
13	32	15	Dec. 9	Apr. 13	125.5±0.40	26.2±0.61	26.2±0.52	5.2±0.11	49.0±1.05	27.5±0.25
14	32	15	do	Apr. 21	132.8±0.74	32.2±0.85	24.4±0.48	4.8±0.12	50.3±0.77	27.2±0.37
15	40	15	do	do	133.3±0.78	38.5±1.04	20.2±0.56	3.0±0.14	41.1±0.79	26.8±0.37
16	50	15	do	Apr. 10	121.8±1.09	26.9±1.27	15.8±0.50	1.8±0.13	44.3±0.66	27.0±0.48
17	32	20	1938 Jan. 13	May 8	115.1±0.47	34.4±0.81	20.0±0.50	4.3±0.13	41.3±0.72	27.8±0.17
18	32	30	Mar. 24	June 18	86.5±0.73	26.5±0.68	18.4±0.46	3.9±0.19	32.8±1.37	25.1±0.30
19	32	45	July 7	Sept. 20	74.6±1.42	15.8±0.62	20.2±0.32	2.6±0.13	40.5±0.95	23.6±0.14
20	32	60	Oct. 20	1939 Jan. 27	99.2±5.80	21.1±0.99	17.0±0.61	2.0±0.17	24.2±0.72	24.4±0.90

HARRISII H<sub>38</sub> (MEAN CIRCUMFERENCE OF BULBS, 7.98±0.23 INCHES)

1 <sup>4</sup>			1937 Aug. 26	1938 Mar. 15	201.0±1.39	48.1±0.57	17.8±0.43	6.2±0.24		30.3±0.44
2	40	5	Sept. 30	Apr. 4	186.2±1.01	43.3±0.79	21.2±0.46	5.4±0.13		30.8±0.33
3	40	10	Nov. 4	Apr. 11	158.3±0.76	36.6±0.76	22.0±0.34	4.7±0.20		32.8±0.38
4	40	15	Dec. 9	May 7	149.7±0.75	43.8±0.87	25.1±0.35	3.7±0.19		34.3±0.27

<sup>1</sup> 25 plants in each lot of *Creole* and 40 plants in each lot of *Harrisii*.

<sup>2</sup> From date of planting in greenhouse.

<sup>3</sup> Mean and standard error.

<sup>4</sup> Bulbs planted in greenhouse on arrival.

<sup>5</sup> Untreated coldframe.

<sup>6</sup> All bulbs up when brought into greenhouse.

A comparison of five pairs of lots stored in moistened peat and dry-soil pack at the same temperatures and intervals (fig. 2) shows that the moist pack induced earlier bloom. From these five pairs the mean difference in time of blooming is 8.66 days, and the odds exceed the 5-percent level and approach the 1-percent level of significance. The individual differences in time of blooming are each statistically significant, but are too small to assume any practical significance except where maximum earliness is desired. These differences in general could have been made up more conveniently by raising the forcing temperature, which is considerably less trouble (although possibly more expensive) than repacking in moist peat. Where maximum earliness is required the two methods could be combined. All the writer's trials thus far have been run at a comparatively cool forcing temperature. An increase of 10° F. during January presumably would have advanced the earliest flowering date (February 24) still further.

Further consideration of the mean dates of bloom in table 3 reveals that lots stored at 32° F. in dry pack, i. e., in original containers, were the last, or among the last, to bloom in each storage-interval group. The differences between storage at 32° dry and that at 50° dry are always significant, but storage at 32° does not always differ significantly in effect from that at 40°. Similar comparison of the data for the number of flowers from these lots shows that 32° storage was always followed by more flowers per plant than 50° storage. The differences between 32° and 40° storage are smaller and not all are significant, but when real differences appear they show more flowers from lots stored at 32°. When *t* tests are applied to four available pairs of measurements, lots stored at 32° and 40° are found to flower significantly later than those at 50°, and lots at 32° exceed those at 50° in number of flowers by a highly significant difference. These data show 32° to be the best of the temperatures tested for holding Easter lilies for long periods with minimum deterioration of forcing quality.

The effect of progressively longer intervals of storage at 32° F. in dry-soil pack is shown in the bar charts in figure 3, plotted from data included in table 3. It appears from the chart that the effect of continued storage in stimulating early bloom is cumulative. The form of the chart suggests a curvilinear relation, in which additional increments of storage have a reduced effect in stimulating earliness. The fact that the suggested curve is obviously not a smooth one is perhaps explainable as due to temperature differences during forcing. The decline in number of flowers shows a similar trend but is less smoothly expressed. The effect of storage at 32° on height is not uniform in trend, but reference to table 3 will show a rather consistent decline in height with progressively increasing periods of 50° dry storage.

The data for days to emergence are also presented in figure 3, *B*, and table 3. All stored lots emerged more promptly than the control lot, and the differences are significant though not large. The mean difference in time of emergence between moist and dry storage at the same temperature and storage duration is 6.63 days, a significant value. The five individual differences are always in favor of moist storage but are not always individually significant. Longer periods of

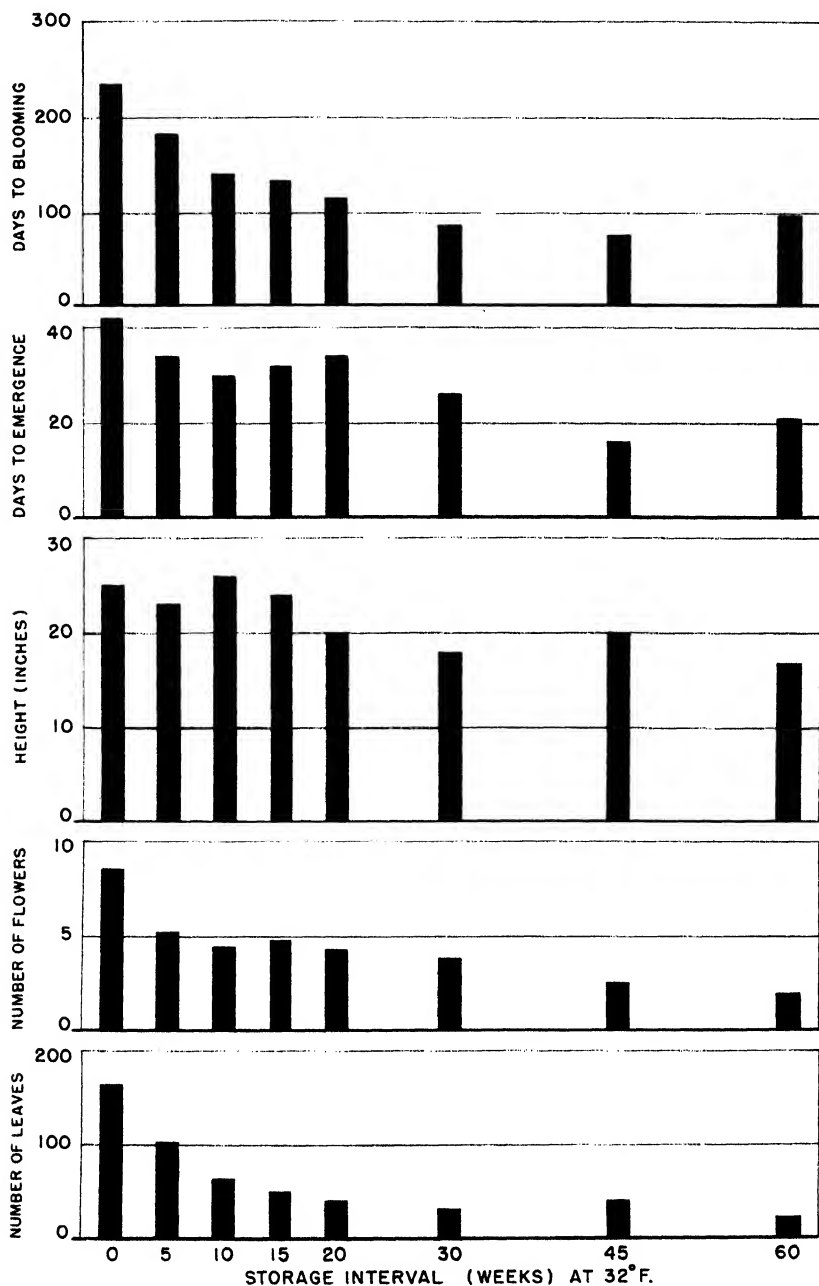


FIGURE 3.—Charts showing effect of duration of storage of the bulbs in dry sandy loam at 32° F. on days to blooming (A), days to emergence (B), height (C), number of flowers (D), number of leaves (E), and relative size of bloom (F), in the Creole Easter Lily in the 1937-38 trials.

dry storage at 40° F. show slower emergence than comparable lots stored similarly at 32° or 50°, but these differences are significant only in the 15-week interval. The *t* test shows no significant differences in days to emerge between 32° and 40°, 40° and 50°, nor 32° and 50° storage when the four available comparisons are grouped. In general, the Creole variety is not difficult to get up, but cool storage and the presence of moisture hasten its emergence.

Decline in height of the plants after storage treatment is notable chiefly in the longer storage intervals at 50° F., but it is also evident after very long storage at 32°.

The number of flowers per plant drops sharply from the control value in all stored lots, the nearest approach to the control appearing in the coldframe lot. This drop in number is more rapid at 40° F. than at 32°, and more rapid at 50° than at 40°, in all comparisons available. A highly significant *t* value is found for the four comparisons between 32° and 50°, but other comparisons, 32° as compared with 40°, and 40° as compared with 50° yield nonsignificant values. Even in 32° storage the decline in number of flowers is the chief limiting factor determining the length of time that bulbs of the 7- to 9-inch size may be held (fig. 3, *D*) without too serious effects in forcing quality.

The number of leaves developed per plant shows a close parallel in decline to the number of days required to bloom and to the number of flowers developed per plant (fig. 3, *A*, *D*, *E*). Again the decline is greater at 50° F. storage than at 32°, greater at 40° than at 32°, and greater with progressive intervals at the same storage. Not all the individual differences are significant, but general trends are evident. Applying *t* tests to four available comparisons, lots stored at 32° show a significantly higher leaf count than lots stored at either 40° or 50°. In addition to the decline in number of leaves, a decline in size of the lower leaves becomes more and more evident with increasing storage intervals.

No measurements of leaf size have been made, but the effect is evident in figures 4 and 5. Bulbs stored 15 weeks or more have incompletely developed lower leaves. Some of those stored for 60 weeks developed leaves irregularly; the lowermost leaves were reduced to scales, and some midway up the stem also remained rudimentary in form.

Size of flower again appears as a relatively stable characteristic, with very few differences approaching the 5-percent level of significance, and no lot differing significantly from the control lot.

The Creole lot which was stored dry for 60 weeks at 32° F. was scattered in the greenhouse, five plants at each of five situations (blocks), to afford a measure of place effects in this particular house. Two lots were placed near the inner end of the house, nearest the steam pipes most frequently used, two lots at the opposite end of the house nearest the outside door, and the fifth lot in the center. On analysis of variance, no significant differences were found between blocks for any of the six characteristics measured. The two blocks nearest the inside door bloomed earlier, but not significantly earlier, than the remaining three blocks. Although there appeared to be a temperature gradient from the inner to the outer end of the greenhouse, the writer failed to demonstrate a significant difference due to position in this trial. It is thought that the place effects measured

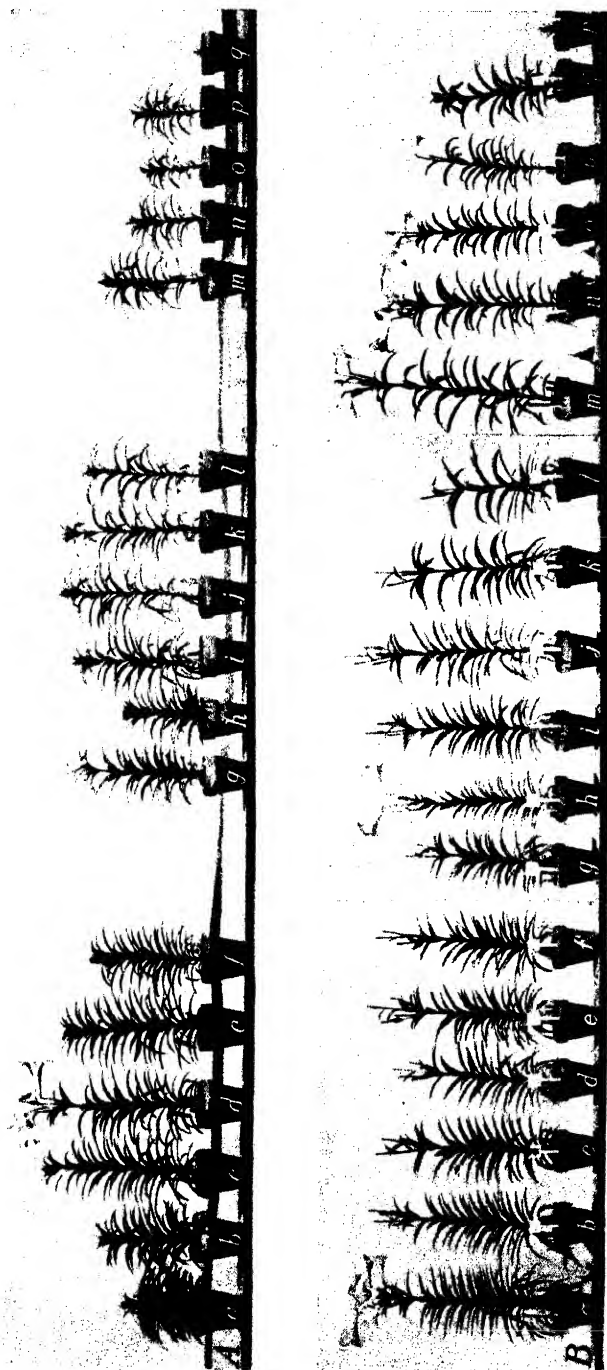


FIGURE 4.—Representative plants from storage experiments with Creole Easter lilies in 1937-38, photographed February 12 (A) and April 23 (B), 1938. Storage treatments in each row: (a) None; (b) 5 weeks in moist pack at 32° F.; (c) 5 weeks in moist pack at 40°; (d) 5 weeks in moist pack at 50°; (e) 5 weeks in dry pack at 32°; (f) 5 weeks in dry pack at 40°; (g) 5 weeks in dry pack at 50°; (h) 10 weeks in coldframe; (i) 10 weeks in moist pack at 32°; (j) 10 weeks in dry pack at 32°; (k) 10 weeks in dry pack at 40°; (l) 10 weeks in dry pack at 50°; (m) 15 weeks in moist pack at 32°; (n) 15 weeks in dry pack at 32°; (o) 15 weeks in dry pack at 40°; (p) 15 weeks in dry pack at 50°; (q) 20 weeks in moist pack at 32°; (r) 30 weeks in dry pack at 32° (planted March 24). Note the trend toward fewer leaves and fewer flowers from the longer storage intervals.



here represent a maximum, since the plants were grown October 20 to February 22, when steam was used regularly and loss of heat through the outside door was greatest, and the lots were so distributed as to include maximum temperature differences in the house. Although the treatments were not randomized through the greenhouse, place effects probably do not seriously affect the general conclusions presented in this paper, but they may account for some of the irregularity in trends shown in figure 3.

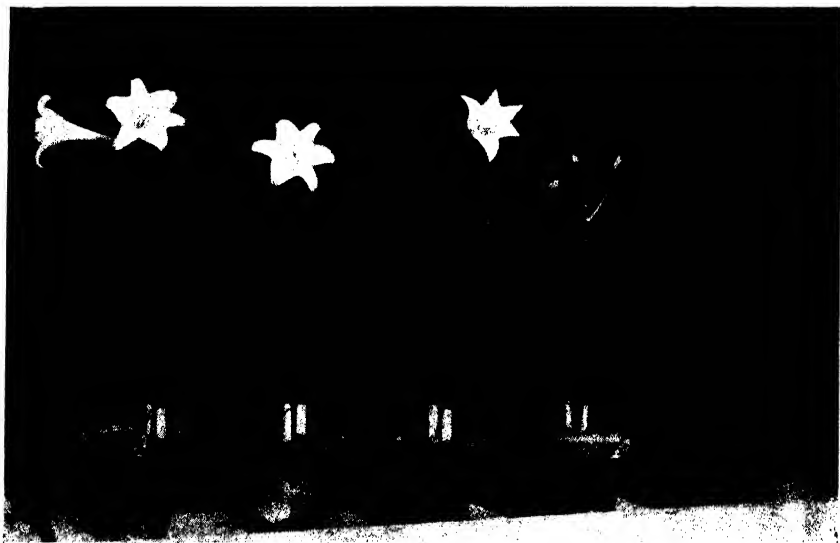


FIGURE 5.—Creole Easter lilies in bloom January 12, 1939, after 60 weeks' storage in dry soil at 32° F. and 94 days in the greenhouse. Note reduced number of leaves and flowers and imperfect leaf development. For comparison, one plant (at extreme right) is from a new crop commercial Creole bulb not subjected to cool storage, after 97 days in the greenhouse.

#### HARRISII VARIETY

The treatments and data for the 1937-38 Harrisii stock are shown in table 3. These data are of interest chiefly because of the differences shown between the Harrisii and Creole varieties when treated alike, and because of certain marked departures in behavior of the Harrisii stock under discussion from that of the Harrisii stock of 1936-37. If the data for Harrisii are compared with those for Creole (table 3), it is found that in untreated lots (lot 1) Harrisii was a month earlier, but after 5 weeks in dry storage at 40° F. Creole was the earlier; after 10 and 15 weeks Creole was again the earlier. Thus Harrisii proved earlier but less responsive to cool storage than Creole in these samples. None of the storage treatments applied to Harrisii (40° dry, for 5, 10, 15 weeks) advanced the actual date of flowering, while Creole exposed to the same treatment flowered in advance of the control after both 5 and 10 weeks' storage, and after 15 weeks it flowered at essentially the same time as the control lot. However, all three intervals at 40° shortened the number of days required to bloom after

planting in both varieties. In *Harrisii* the number of flowers per plant declined steadily with progressively longer storage intervals, with all differences significant. After comparable storage intervals the number of Creole flowers dropped sharply after 5 weeks' storage, remained unchanged after 10 weeks, then dropped lower after 15 weeks. In *Harrisii* the size of flower increased steadily with increasing time in storage; in Creole no such change was evident. Incidentally, the significant increase in *Harrisii* is believed to be due not to storage, but to environmental factors that became successively more favorable for growth and development as these lots flowered. Such comparisons between these two varieties are probably sound, since the two presumably matured and were harvested at the same time and were without storage treatment before arrival. It is still conceivable that the environmental effects of the two production areas were responsible for the differences observed, but this evidence is the best the writer has obtained thus far to show that commercial stocks differ in capacity to respond to cool storage.

A comparison of the *Harrisii* stock of 1936-37 (table 2) with that of 1937-38 (table 3) shows some striking differences. The 1936-37 stock was purchased through a New York dealer and reached Beltsville one month later in the season than the 1937-38 stock, which was bought direct from a producer in Bermuda. The bulbs in the two lots were of the same size and grade, and received similar treatment by the writer except that more varied treatments were imposed on the earlier lot. The 1937-38 untreated lot emerged much more promptly, bloomed earlier, and produced twice as many flowers per plant as the corresponding lot of the preceding year. The response to storage was similar in the 2 years; in neither year did any stored lot bloom before the control lot. It seems probable that the 1936-37 stock of *Harrisii* received some unfavorable treatment before reaching Beltsville. Since the evidence presented above indicates that *Harrisii* is somewhat divergent from other Easter lilies in response to storage, it is possible that the variety suffers adverse effects from routine bulb-storage practices.

#### CORRELATION AMONG THE CHARACTERISTICS MEASURED

Cool storage is utilized chiefly to advance or retard the date of flowering in Easter lilies, but a number of other characteristics recorded in this study such as number of leaves and number of flowers, appeared to be strongly modified by the treatments. It is of considerable interest to learn how closely these characteristics are correlated, or how strong is their tendency to respond together to storage treatments. Simple correlation coefficients have been computed for all possible pairs of the seven characteristics measured in the Creole variety and of the six measured in the *Harrisii* variety in the 1937-38 trials.

Because of the large number of degrees of freedom, even very low correlation coefficients have statistical significance; however, comparatively few pairs of characteristics show close association. The square of the value of  $r$  is sometimes used as an index of percentage correlation. For example, in the Creole variety, 52 percent of the variation in height, 37 percent of that in number of flowers, 77 percent of that in number of leaves, and 30 percent of that in duration of bloom are found associated with days to blooming. Consequently

treatments designed to accelerate bloom may be expected to reduce the number of leaves, and they are also likely to reduce height, number of flowers, and duration of bloom. Number of flowers shows a 57-percent correlation with number of leaves. Few of the other relationships are sufficiently close to assume practical importance. Size of the flower shows little or no correlation with other factors studied in the Creole variety.

The *Harrisii* variety included only 160 recorded individuals, and less varied treatments were imposed on these than on Creole. Few of the correlation values are high, although many are significant. Some differences in varietal tendency appear. Accelerated flowering is associated to the degree of 25 percent with taller plants in *Harrisii*, but to 52 percent with shorter plants in Creole. There is a 25-percent tendency for late-blooming plants of *Harrisii* to bear larger flowers, but no relation at all appears between days to bloom and size of flower in Creole. As explained above, there is some reason to believe that *Harrisii* displays different responses to storage from those of Creole, but the differences found here may result chiefly from sampling during part of the calendar year with *Harrisii* (March to May) and during the entire year (February to January) with Creole.

#### 1938-39 EXPERIMENT

No formal storage trials were planned for the season of 1938-39 but the performance of one lot stored and forced for early vegetative growth for other purposes seems of sufficient interest to report. Seedling Easter lilies were dug at Charleston, S. C., on July 13, 22 months from seed. At this time some of the tops were well matured and some still green. A random selection of five seedlings from each of five progenies made up the experimental lot. The bulbs were shipped to Beltsville, Md., and stored in moistened peat at 50° F. from July 22 to September 2, or 6 weeks. On the latter date they were potted into 4- or 6-inch pots according to size, and were forced under the conditions of previous trials. A large number produced flowers at far earlier dates than any lot of new crop Easter lilies previously forced. The range of flowering dates was December 10 to January 17 for the 25 bulbs from which data were collected. The mean flowering date was January 2. The plants required  $121.4 \pm 2.25$  days to bloom, and bore  $2.6 \pm 0.32$  flowers per bulb, at a height of  $18.3 \pm 1.24$  inches above the soil.

These data suggest that Easter lilies for December and January bloom should be produced from new crop bulbs rather than from stored bulbs of the previous season, provided the new crop bulbs can be prepared safely and consistently. The new crop lilies compared very favorably with stored Creoles of the previous season. At least four factors may be utilized in inducing maximum earliness of bloom in new crop Easter lilies: Genetic earliness, early digging, cool storing, and forcing at comparatively high temperatures. Considerably more detailed information on the hazards involved and on the most efficient combination of these factors is needed before new crop lilies will appear on the market at Christmas, but this objective should be attainable.

## DISCUSSION

The control of flowering time in bulbous plants by suitable manipulation of storage temperatures has long been in commercial use. Workers at the Wageningen and Lisse laboratories in the Netherlands have made detailed studies of the seasonal changes in bulbs of hyacinth, tulip, and narcissus.<sup>2</sup> The Netherlands workers have determined the temperature requirements for organ formation and for elongation in these bulbs, and have then devised temperature sequences for early and for late forcing.

Pfeiffer<sup>3</sup> studied the successive stages of development of the floral axis in bulbs of Bermuda Harrisii lilies stored at 10° to 13° C. (50° to 55.4° F.) and in Japanese Giganteum stored at 3° C. (37.4° F.). Harrisii bulbs received in August were in the vegetative stage, which persisted to mid-October when the predifferentiation stage of the terminal bud was recognizable. In the variety Giganteum, bulbs received in December already showed evidence of the predifferentiation stage, but floral organs were not recognizable in storage until April 16. Development in storage was slow in both varieties, but the cumulative effect of this development brought more rapid completion of the flower primordia after planting and hence more prompt blooming.

No morphological study of the Creole variety, which was used chiefly in the present study, is available. It may be assumed tentatively; however, that the cool storage treatments were applied mainly prior to differentiation of the floral organs. The early stages of bud development and possibly also the later stage of organ formation may have an optimum temperature near 50° F. The observed advantage of 50° over 32° storage, and some of the differences in performance of the several varieties and of different stocks of the same variety may thus be explainable on the basis of the temperature requirements of successive developmental phases within the bulbs.

Previous studies of the effect of cool storage on the performance of Easter lilies replanted in the field have been made in Bermuda<sup>4</sup> and in Florida.<sup>5</sup> In the Bermuda experiment, bulbs of the Harrisii variety were subjected to 37° F. storage in sand for monthly periods ranging from 0 to 4 months, beginning September 18, about 8 or 10 weeks after harvest. The bulbs subjected to cool storage emerged and bloomed more promptly but produced fewer flowers than controls planted at the start of the storage trial; there was an actual advance over the controls in the flowering date of the lots stored 1 and 2 months. The authors of the Bermuda report stated that the decline in number of flowers per bulb resulted from ordinary storage as well as from cool storage. Shippy<sup>5</sup> found that the Florida strain of Easter lily (similar to Creole and perhaps identical with it) emerges and blooms earlier in Florida field culture after a period of cool storage. A temperature of 40° F. and a packing of dry sand were used. Shippy varied the

<sup>2</sup> PURVIS, O. M. RECENT DUTCH RESEARCH ON THE GROWTH AND FLOWERING OF BULBS. I. THE TEMPERATURE REQUIREMENTS OF HYACINTHS. *Sci. Hort.* [Wye, Kent] 5: 127-140, illus. 1937.

— RECENT DUTCH WORK ON THE GROWTH AND FLOWERING OF BULBS. II. THE TEMPERATURE REQUIREMENTS OF TULIPS AND DAFFODILS. *Sci. Hort.* [Wye, Kent] 6: 160-177, illus. 1938.

<sup>3</sup> PFEIFFER, NORMA E. DEVELOPMENT OF THE FLORAL AXIS AND NEW BUD IN IMPORTED EASTER LILIES. Boyce Thompson Inst. Contrib. 7:311-321, illus. 1935.

<sup>4</sup> BERMUDA DEPARTMENT OF AGRICULTURE. AN EXPERIMENT ON COLD STORAGE OF LILY BULBS. Bermuda Dept. Agr., Agr. Bul. 14: 52-54. 1935.

<sup>5</sup> SHIPPY, WILLIAM B. FACTORS AFFECTING EASTER LILY FLOWER PRODUCTION IN FLORIDA. Fla. Agr. Expt. Sta. Bul. 312, 19 pp., illus. 1937.

digging date and the date and duration of cooling, and included warm storage treatments and bulbs replanted immediately as controls. Bulbs were accelerated in emergence and flowering by cool storage for an interval of 30 days or more but not for a 21-day interval. A few flowers were produced in December, and more in January, from cool-stored bulbs replanted in the field. The most favorable effects in the production of early bloom followed cool storage of 30 days or more applied after the middle of August. Shippy found that fewer flowers were produced from cool-stored lots, but that the increase in number of bulbs was not similarly affected.

The present investigation indicates that 50° F. is more effective in inducing early bloom than lower temperatures, and that moistened peat is slightly superior to dry-sand pack for this purpose. It also appears that all possible factors subject to control and tending to early bloom have not yet been combined. A possible danger in digging before bulbs are sufficiently mature is suggested in one of the writer's tests not here detailed.

However, early bloom is only one goal of Easter lily forcing. It is necessary to provide Easter lilies in every month of the year in response to market demand. The present study shows that this can be done with a single variety and a single digging date, but suggests that better ways should be found for producing fall bloom. After Easter lily bulbs are held for long periods in the least deleterious storage condition known (32° F. dry), they are inferior in forcing performance. Commercial Creole bulbs of the 7- to 9-inch size can be brought to flower from late February to September by simple manipulation of the conditions of storage, and the plants and flowers will be of satisfactory grade. Bulbs of larger size, commonly used for long storing in the trade, will probably produce satisfactory bloom from October to December. Further research on the effects of digging date and forcing temperature should lead to safe provision for November to January bloom from new crop bulbs. The period from September to November probably must be covered by bulbs stored nearly a full year, the practice now followed with Japanese Giganteum. Bulbs of the larger sizes should be used, as is customary when long storage is planned. Present commercial practice now utilizes Erabu for early bloom and Giganteum for late bloom. Although much of the year can be covered with a single variety such as Creole, it is perhaps doubtful whether a single variety can be made to perform most satisfactorily over the whole period.

#### SUMMARY

Storing Easter lily bulbs for 5 or more weeks at 32° to 50° F. accelerates flowering. Storage at 50° is more effective than at 40° or 32° in producing this effect. Bulbs packed in moistened peat moss during the storage interval flowered slightly in advance of those packed in dry soil or sand.

Storage for 10 weeks or more at 50° F. is distinctly deleterious, reducing the number of flowers and the number of leaves per plant. Similar effects result from storage for longer intervals at 40°. Storage at 32° in dry sand kept Creole Easter lilies with less deleterious effects on forcing quality than at 40° or 50°. After 45 weeks at 32° the

quality was barely acceptable, and after 60 weeks the average performance of 7- to 9-inch bulbs was poor.

Effects of cool storage on time required for the plants to emerge from the soil and on the height of the plant are less consistently evident, but when differences appear they are in the direction of decline from the level shown by control plants not subjected to storage. The size of the flower is a relatively constant characteristic of the variety, and is little affected by storage of the bulbs.

It appears probable that new crop bulbs of early varieties can be forced for Christmas by suitable combination of early harvest and cool storage treatment.

Cool storage of Easter lily bulbs is a convenient primary control over time of flowering. The customary control factor, forcing temperature, can be used as a fine adjustment after this primary control has been applied.



# A STUDY OF VARIOUS METHODS OF PRESERVING LEGUMES AND OTHER FORAGES BY ENSILING<sup>1</sup>

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## INTRODUCTION

During the past 20 years there have appeared three principal methods of preserving legumes by ensiling. These are the A. I. V. process developed by A. I. Virtanen (3, 37, 38),<sup>2</sup> the molasses method first used at the Kansas Agricultural Experiment Station (27, 33, 34), and the phosphoric acid method that seems to have been first proposed by Virtanen (37). The present writers have been interested in the preparation of silages from a large variety of forage crops by the above methods and by a number of other less well-established procedures.

In order to decide whether a particular procedure was suitable for trial on a silo scale it has been the practice at this station to try out such a procedure first on bottle or barrel lots. The results of the preliminary trials proved to be very reliable guides as to what might be expected on a larger scale. For example, alfalfa put up by the A. I. V. method has invariably been good in bottles, barrels, or silos. The same has been true of alfalfa silage made with phosphoric acid or molasses when these were used at the rate of 30 and 60 pounds per ton, respectively. Poor silage has been the rule, irrespective of the type of container, when no preservative was added. With bottle or barrel lots particular care must be observed to pack the forage tightly so as to exclude air.

The silages were analyzed for dry matter, pH value, carotene, and in some cases ammonia and total nitrogen as an additional check on preservation. The analyses of most interest are pH value, which is a good criterion of preservation, and  $\beta$ -carotene, which is necessary for the production of winter milk of high vitamin A potency.

In cases where sufficient material was available, feeding tests were made with cows of the University herd to determine the palatability of the silage.

## PREPARATION OF SILAGES

### BOTTLE SILAGES

The bottle silages were prepared by mixing by hand 1 kg. of the freshly cut and chopped forage with the appropriate amount of preservative. A quart milk bottle was filled with the material and closed with a rubber stopper containing a Bunsen valve. The material must be pressed in very tightly with a round flat-end stick, such as a hammer handle, to prevent mold growth. With material

<sup>1</sup> Received for publication August 9, 1940. Presented in part before the Division of Agricultural and Food Chemistry of the American Chemical Society at the September 1940 meeting at Detroit, Mich.

<sup>2</sup> Italic numbers in parentheses refer to Literature Cited, p. 346.



containing 25 percent or less of dry matter, an index of good packing is the accumulation of juice on top of the material. A bottle holds approximately 750 gm. of silage. Table 1 gives the analytical results on the bottle silages.

TABLE 1.—*The effect of various preservatives on the composition and quality of silage in bottle experiments*

Lot No. <sup>1</sup>	Material <sup>2</sup>	Treatment	Preservative added per ton	Dry-matter content	pH value	Carotene per gram of dry matter	Quality of silage <sup>3</sup>
				Per-cent		γ	
5-9	Alfalfa (5)	None		25.3	5.1	168	Poor.
5-0	Alfalfa	A. I. V.	70 liters of 2N acid.	27.2	4.0		Good.
8-5	do	Corn meal	30 pounds	22.2	5.5	244	Poor.
8-7	Alfalfa (2)	do	60 pounds	22.0	5.3	182	Do.
8-9	Alfalfa	do	90 pounds	24.2	5.3	231	Do.
8-11	Alfalfa (2)	do	120 pounds	25.2	4.6	174	Fairly good.
9-37	Alfalfa	Soured-whey concentrate.	70 pounds	35.5	4.5	190	Do.
9-35	do	do	140 pounds	32.0	4.5	207	Good.
9-33	do	do	280 pounds	32.5	4.3	200	Do.
9-28	do	Sulfamic acid	50 pounds	28.0	4.6	181	Fair.
8-65	do	do	80 pounds	20.5	4.5	208	Do.
8-64	do	do	160 pounds	21.0	3.4	327	Do.
8-1	do	Whey powder	20 pounds	24.6	4.7	225	Poor.
8-2	do	do	80 pounds	24.3	4.3	207	Good.
6-0	Alfalfa and corn (1-1)	None		23.0	4.4	Trace	Fairly good.
6-3	Alfalfa and rye (1-1)	do		21.0	4.6		Fair.
6-2	Corn	do		20.0	3.7		Good.
9-13	Clover and timothy (2).	do		24.7	5.6	241	Poor.
9-26	do	A. I. V.	70 liters of 2N acid.	25.0	3.9	156	Good.
9-16	do	Phosphoric acid	30 pounds	26.5	4.0	212	Do.
9-20	Clover and timothy	Molasses	70 pounds	25.0	4.3	208	Do.
9-22	do	Soured molasses	60 pounds	26.0	4.0	210	Do.
8-14	Clover (red)	A. I. V.	70 liters of 2N acid.	25.0	3.9	68	Do.
8-15	do	Phosphoric acid	30 pounds	26.1	4.0	102	Do.
8-16	do	Molasses	70 pounds	26.5	4.2	80	Do.
8-17	Grass (lawn)	A. I. V.	70 liters of 2N acid.	24.5	3.9	196	Do.
8-18	do	Phosphoric acid	30 pounds	25.0	4.1	162	Do.
8-19	do	Molasses	70 pounds	26.2	4.3	152	Do.
9-1	Oats and peas	None		18.2	4.5	340	Fair.
9-11	do	A. I. V.	70 liters of 2N acid.	16.5	3.8	371	Good.
9-4	do	Phosphoric acid	30 pounds	17.0	3.9	363	Do.
9-8	do	Molasses	70 pounds	18.0	4.0	371	Do.
6-18	Sweetclover	None		35.5	4.5		Poor.
6-19	do	Molasses	40 pounds	35.5	4.3		Good.
6-20	do	do	100 pounds	35.5	4.2		Do.
7, 8-0	Sudan grass (2)	None		20.2	5.0		Poor.
7-1	Sudan grass	A. I. V.	50 liters of 2N acid.	24.0	4.4		Good.
7-2	do	Molasses	70 pounds	26.8	4.1		Do.

<sup>1</sup> To aid in comparing various lots, the first number refers to the year and the second to the sample, e. g., 5-9 refers to 1935 bottle No. 9.

<sup>2</sup> Figures in parentheses denote the number of samples averaged.

<sup>3</sup> Quality based on appearance, odor, and chemical analyses.

#### BARREL SILAGES

The barrel silages were prepared as follows: 200 pounds of the freshly cut and chopped forage were mixed with the correct amount of preservative and the mixture was put into a wooden barrel. The material was well tramped down during the filling. The upper hoops were left off the barrel during the packing, and after the barrel was tightly packed to above the top the head was forced in and the hoops were replaced. Table 2 gives the analytical data on the barrel silages.

TABLE 2.—The effect of various preservatives on the composition and quality of silage in barrel experiments

No. <sup>1</sup>	Material <sup>2</sup>	Treatment	Preservative added per ton	Dry-matter content	NH <sub>3</sub> -N as proportion of total N	pH value	Carotene per gram of dry matter	Quality of silage
5-0	Alfalfa	None		Percent 21.6	Percent 45.0	5.0	7	Poor.
5-10	do	do		22.6		4.5	65	Do.
6-0	do	do		31.1	18.1	4.6	158	Fair.
7-1	do	do		23.5	8.8	4.6	164	Do.
8-1	do	do		23.2	7.4	4.3	162	Good.
8-10	do	do		26.2	14.5	4.7	130	Poor.
9-1	do	do		25.2		5.3	77	Do.
9-2	do	do		26.0		5.3	18	Do.
7-14	do	Wilted, 27 percent dry matter		27.3	13.4	4.9	133	Do.
8-3	do	Wilted, 28 percent dry matter		27.8	11.6	4.9	132	Do.
7-17	do	Wilted, 33 percent dry matter		27.8	9.4	4.9	109	Do.
8-5	do	Wilted, 38 percent dry matter		37.7	14.5	4.8	125	Do.
9-6	do	Wilted, 42 percent dry matter		42.0		4.9	176	Do.
9-8	do	Wilted, 46 percent dry matter		46.4	9.1	4.5	126	Fair.
6-7	do	Wilted, 48 percent dry matter		44.0		4.6	103	Do.
6-7-0	Alfalfa (5)	A. L. V.		26.4		3.7	200	Good.
5-1	Alfalfa	Molasses	10 pounds	21.6	5.0	5.2		Poor.
5-2	do	do	20 pounds	21.6	35.2	5.2		Do.
5-7	do	do	40 pounds	21.6	21.9	5.2		Do.
6-9	Alfalfa (5)	do	60 pounds	27.5	8.1	4.1	138	Good.
6-7	Alfalfa (4)	do	80 pounds	26.9	8.3	4.1	156	Do.
5-6	Alfalfa (2)	do	100 pounds	30.1	6.9	3.9	157	Do.
5-3	do	do	200 pounds	27.5	6.9	3.8	148	Do.
7-16	do	do	280 pounds	21.6	4.1	3.7	146	Do.
8-4	do	Molasses, wilted, 27 percent dry matter	80 pounds	28.4	8.2	3.9	173	Do.
7-19	do	Molasses, wilted, 28 percent dry matter	60 pounds	29.9	11.6	4.1	164	Do.
7-18	do	Molasses, wilted, 32 percent dry matter	90 pounds	32.0	6.3	4.0	175	Do.
8-7	do	Molasses, wilted, 38 percent dry matter	80 pounds	32.4	5.0	4.1	120	Do.
7-15	do	Molasses, wilted, 42 percent dry matter	60 pounds	34.5	8.0	4.2	135	Do.
8-6	do	do	80 pounds	36.4	4.2	3.9	143	Do.
9-7	do	do	60 pounds	38.7	8.3	4.1	142	Do.
	do	Molasses, wilted, 42 percent dry matter	40 pounds	42.0		4.6	174	Poor.

<sup>1</sup> To aid in comparing various lots, the first number refers to the year and the second to the sample, e. g., 5-0 refers to 1935 barrel No. 0.<sup>2</sup> Figures in parentheses denote the number of samples analyzed.<sup>3</sup> Quality based on appearance, odor, and chemical analyses.

TABLE 2.—The effect of various preservatives on the composition and quality of silage in barrel experiments—Continued

No.	Material	Treatment	Preservative added per ton	Dry-matter content	NH <sub>3</sub> -N as proportion of total N	pH value	Carotene per gram of dry matter	Quality of silage
9-9	Alfalfa	Molasses, wilted, 46 percent dry matter.	30 pounds	Percent 45.8	Percent	4.5	7	Fair.
6-2	do	do	40 pounds	45.5	6.7	4.2	70	Good.
6-6	do	do	80 pounds	47.0	6.5	4.1	103	Do.
7-12	do	Phosphoric acid	100 liters of 2N acid	25.6	4.6	4.1	114	Do.
7-13	do	do	150 liters of 2N acid	26.4	2.7	3.7	123	Do.
7-9-4	Alfalfa (2)	Whey powder	40 pounds	26.8	6.1	4.2	173	Do.
7-9-5	do	do	80 pounds	28.1	5.4	4.0	172	Do.
9-10	Alfalfa	Corn meal	40 pounds	26.5	5.0	5.0	174	Do.
9-12	do	do	80 pounds	26.8	5.0	4.9	54	Poor.
9-14	do	do	120 pounds	27.7	—	4.6	42	Do.
9-11	do	do	40 pounds, plus H <sub>2</sub> SO <sub>4</sub> , 2 pounds	22.3	—	4.6	116	Fair.
9-13	do	do	80 pounds, plus H <sub>2</sub> SO <sub>4</sub> , 2 pounds	23.1	—	5.0	115	Poor.
9-15	do	do	120 pounds, plus H <sub>2</sub> SO <sub>4</sub> , 2 pounds	26.6	—	4.8	139	Do.
9-16	do	Sodium chloride	10 pounds	21.1	—	4.5	137	Fair.
9-17	do	do	20 pounds	23.3	—	5.5	170	Poor.
7-8-0	do	do	135 grams	23.7	—	5.0	185	Do.
6-3	Alfalfa (2)	Phosphorus pentasulfide	—	23.7	19.5	4.9	102	Do.
6-4	Alfalfa and canary grass (1-1)	None	—	28.8	9.6	4.4	190	Good.
6-4	Alfalfa and quackgrass (1-1)	do	—	29.5	14.2	4.4	156	Poor.
6-21	Alfalfa and timothy (1-1)	do	—	34.0	—	4.3	169	Good.
6-22	Clover and timothy (2), (1-1)	do	—	23.6	—	4.3	207	Fair.
8-18	Alfalfa hay and pea vines (1-5)	Molasses	30 pounds	23.6	13.0	4.5	90	Good.
8-16	do	do	60 pounds	18.6	—	4.1	80	Do.
8-17	do	do	30 pounds	10.4	—	3.9	84	Do.
6-9-22	Soybeans (2)	None	60 pounds	24.8	—	3.9	126	Poor.
6-5	Soybeans	A. I. V	—	25.4	22.0	5.4	112	Good.
9-21	do	Molasses	40 pounds	30.6	7.4	4.0	127	Poor.
9-20	do	do	60 pounds	30.7	—	5.2	124	Do.
9-19	do	do	80 pounds	31.3	—	5.1	118	Do.
6-9-18	Soybeans (2)	do	100 pounds	28.6	7.8	5.0	112	Good.

## SILAGE LOTS

Small lots of from 5 to 10 tons were put up in silos, two to six different treatments in a silo. Each lot was marked by wooden strips. Table 3 summarizes the analytical data on these silages.

TABLE 3.—*Effect of various preservatives on the composition and quality of silage in silo experiments*

Year	Material	Treatment	Preservative added per ton	Dry-matter content	NH <sub>3</sub> -N as proportion of total N	pH value	Carotene per gram of dry matter	Quality, appearance, and odor of silage	Order of palatability <sup>1</sup>
1938	Alfalfa	Faiyagina method	Pounds	Percent	Percent				
	do	Fresh as ensiled		26.3	5.6	5.6	19	Poor	
	do	Molasses		27.0	27.0	105	105	Good	
1939	Sudan grass	Fresh as ensiled		26.4	4.2	4.2	135	Good	
	do	Whey powder	30	30.6	4.4	4.4	137	Fairly good	
1939	Alfalfa	Willed, 40 percent dry matter		25.5	8.2	4.6	126	Poor	2
1939	do	Corn meal	150	27.8	8.2	4.5	90	Fairly good	5
1939	do	None		25.5	17.8	5.3	121	Poor	1
1939	do	Sodium chlorate	5	25.3	10.4	4.3	192	Good	4
1939	do	Fresh as ensiled		25.0	4.4	3.6	185	Good	3
1935 to 1937	do	A. I. V. (14)		24.5	7.5	4.1	200	Good	(2)
1935 to 1940	do	Molasses (15, 16)	65	25.8	9.7	4.1	212	do	(2)
1937 to 1940	do	Phosphoric acid (16)	8.3	25.3	4.3	4.3	130	do	(2)

<sup>1</sup> Nos. 1 and 2 were very palatable and distinctly superior to the others. No. 5 was not liked by the animals. The tests were based on the time required to consume the silage at each feeding. The most palatable silage was completely consumed in half an hour, while the less palatable silages were consumed only after several hours.

<sup>2</sup> These silages were all of good palatability.

## EXPERIMENTAL RESULTS

## NO PRESERVATIVE ADDED

The various legume forages, when put up with no preservative added, gave variable results (tables 1, 2, 3). The bottle silages were mostly poor. The best samples from the barrel silages were those that had been very well packed. In 1939 the packing was not so well done and much poorer silage was obtained. Although the alfalfa with no preservative was well down in the silo with 20 tons of other silage on top of it, still it was poor. While it is possible on occasion to get good silage from fresh material without added preservative, the procedure is not to be recommended.

Cooper (6) found that spoilage almost invariably resulted when green alfalfa was put up without added preservative. This has also been reported by Swanson and Tague (34), Schieblisch (31), Newlander et al. (24), Politi (26), and others. Lavenir and Chaudet (19) state that alfalfa put up with no preservative gives variable results, but even in the "good" silage they found 75 percent of the nitrogen in the form of water-soluble compounds and almost entirely amino nitrogen.

On the other hand Neidig and Snyder (23) found that sweetclover (cf. table 1) gave silage of excellent quality without the use of a preservative. Huffman (15) summarizes the results as follows:

Some recent investigations indicate that good legume silage can be made without any additions, and that exclusion of air is the main factor to be considered.

## PRESERVATIVES ADDED

## ACIDS AND MOLASSES

The A. I. V. and phosphoric acid procedures gave generally good results. The molasses silages improved with increasing amounts of molasses up to approximately 3 percent for alfalfa and 5 percent for soybeans. No difference was found between the use of cane and of beet molasses, nor between diluted and undiluted molasses as preservatives. All gave uniformly good results. These silages will not be considered further in this paper, as more complete data on other lots are given elsewhere (14, 16).

## WHEY POWDER

Bottle silage treated with powdered whey at the rate of 80 pounds per ton was good, but 20 pounds per ton was not enough. Barrel silage prepared with whey powder at 40 pounds per ton was good, but that with 80 pounds was better. In the silo 30 pounds gave fair results. Thus it seems that 30-40 pounds of whey powder per ton are required to make good alfalfa silage.

## SOURD-WHEY CONCENTRATE

Sourd-whey concentrate was prepared as follows: 3,600 cc. of whey was incubated for 48 hours with *Lactobacillus bulgaricus*, at which time it had an acid content of 0.74 percent calculated as lactic acid. This solution was concentrated under vacuum to 600 cc. and a lactic acid content of 4.44 percent. This concentrate was added at the rate of 35, 70, and 140 gm. per kilogram of forage, which corresponds to 420, 840 and 1,680 pounds of liquid whey per ton. (Whey powder at 40 pounds per ton corresponds approximately to

660 pounds of unconcentrated whey per ton.) All samples of soured whey produced good silage, and there was a slight improvement in quality as the amount was increased.

Samarani in 1913 (29) proposed the addition of dilute lactose solution as a fermentable carbohydrate in the preparation of grass silage. Whey has been proposed by a number of workers (1, 9, 28, 39), but has not given promising results because of its low sugar content. However, very small amounts of whey, of the order of 200 pounds per ton, were used. Wildt (39) found no advantage in using whey soured by *Streptococcus lactis*. Allen, Watson, and Ferguson (1) obtained good silage with whey added at the rate of 150 gallons per ton (1,360 pounds per ton) and with dried whey added so as to supply 1 pound of lactose per 100 pounds of green forage (about 28 pounds of whey powder per ton). In the case of the dried whey, they found it difficult to obtain even distribution; no such difficulty was experienced in the work reported here.

#### CORN MEAL

Bottle silages prepared with amounts of corn meal from 30 to over 100 pounds per ton (table 1) were poor, and with 120 pounds were only fair. The same result was found with the barrel lots (table 2). The addition of 0.1 percent of sulfuric acid (calculated on the basis of fresh forage) improved the silage somewhat, especially as to the preservation of carotene. In the silo lot, 150 pounds of corn meal per ton gave fairly good silage, but the pH value was still slightly high and the carotene preservation was rather low. However, this silage was very palatable and seemed to show that good alfalfa silage can be made if 150 pounds per ton or over are used.

Swanson and Tague in 1917-18 (34) reported good silage with 200 pounds of corn meal per ton of fresh forage. They also tried germinated corn and found that it produced better results than ungerminated corn.

#### SULFAMIC ACID

Sulfamic acid was used in the preparation of some bottle silages, and 50 to 160 pounds of 2.5 N acid per ton gave well-preserved silage. However, it always had a peculiar and rather disagreeable odor.

#### SOURD MOLASSES

Clover-timothy forage was put up in bottles with molasses that had been diluted 1-3 and soured with *Lactobacillus delbrückii* to pH 4.07 and a lactic acid content of 2.1 percent. This material was added at the rate of 180 pounds diluted or 60 pounds undiluted molasses per ton and gave good preservation and good silage. It was somewhat better than that put up with unsoured molasses.

#### SALT

Barrel silages were put up with 0.5 and 1.0 percent of sodium chloride, i. e., 10 and 20 pounds per ton, but were poorly preserved in both cases. On the other hand, the silage made in the silo with 5 pounds sodium chloride per ton was well preserved but only fairly palatable (table 3).

Wyant in 1920 (44) tried the addition of 1 percent of salt to alfalfa and obtained fairly good silage. Kuckler and Wachter (18) used 0.5 percent of salt and 0.5 percent of sugar with good results, while Luchetti (20) states that even 0.1 percent of sodium chloride favors the development of acidity. Virtanen (38) states that some salt is generally added to the fodder in making ordinary (as distinct from A. I. V.) silage in Finland. The usual amount is from 0.5 to 1 percent of sodium chloride. Analyses are given by him for 98 such samples. Practically all were of poor quality with pH values above 4.6 and ammonia nitrogen amounting to more than 15 percent of the total nitrogen.

#### PHOSPHORUS PENTASULFIDE

The phosphorus pentasulfide was burned in the barrel until the barrel was filled with fumes, and then the chopped alfalfa was packed into the barrel. The results were poor, especially as regards carotene preservation (table 2).

#### SOME OTHER METHODS OF ENSILING

##### USE OF WILTED FORAGE

Barrel lots were put up with alfalfa that had been wilted to varying degrees, from approximately 27 to 48 percent dry matter. From table 2 it can be seen that poor results were obtained when no preservative was added. The best results were with 46 and 48 percent dry matter. With added molasses good silage was again obtained, but there was no advantage over the unwilted alfalfa. There were disadvantages in wilting, e. g., loss of nutrients, carotene, etc. In the silo the alfalfa that was wilted to 40 percent dry matter gave a very moldy and unpalatable silage, which may have been due to the fact that wilted dried alfalfa does not pack well (17, 25). However, this lot of silage was down in the silo below 10 tons of good silage. Also, as seen in table 3, this silage had a low carotene content. This method of ensiling does not seem advisable as a practical farm procedure.

Cooper (6) in 1917 stated that good legume silage could be made if the material was very dry, finely cut, and tightly packed. Eckles (7) found that 40 percent dry matter permitted good preservation, while Schieblich (31) found it possible to produce excellent alfalfa silage by first drying to 35 percent dry matter. At 15 to 21 percent dry matter, good silage was not obtained unless preservative was added. Bender and others (2) found that material high in dry matter (45 to 67 percent) showed considerable charring or molding, while that with 25 to 40 percent dry matter did not; with lower percentages of dry matter putrefaction took place. The high temperature of low-moisture silage could be reduced if sufficient pressure was developed to exclude the air. Similar results as to the possibility of producing silage by partial drying have been reported by many workers (8, 13, 24, 25, 32, 33, 34, 39, 42, 43).

Mikhin et al. (21) found that green silage with 45 to 50 percent dry matter developed only one-half to one-third the acidity of normal silage. Shepherd and Woodward (32, 41, 42) have reported good silages with dry-matter content from 30 to 77 percent and pH values from 4.7 to well over 5.0. The writers have found that good silage (as to palatability, odor, appearance, etc.) always has a pH value

well below 4.5. Nehring (22) stated that at pH 4.25 or lower the silage was good and free from butyric acid, at values from pH 4.5 to 4.7 the silage was poor, and above pH 4.8 butyric acid was always present and the silage was spoiled. Brouwer (4) reports that silage with a pH value above 4.5 is poor, from 4.5 to 4.0 fairly good, and from 4.0 to 3.5 good; and that for practical purposes, a simple examination of pH, odor, and appearance is sufficient to distinguish good silage.

Taylor (35) reports, as do Shepherd and Woodward (32, 42), that wilting of the green material prior to ensiling reduced the carotene content to a marked degree. Ellenberger and others (8, 36) state that while grasses or legumes will make good silage without further addition if the moisture conditions are just right, under ordinary farm conditions it is better to use molasses.

A method of storing partly dried forage in silos which is used in Italy has been described by Samarani (30) and by others (5, 11, 12, 26). Fred and Peterson (40) tested this method of ensiling alfalfa. The material was dried to 55 to 70 percent dry matter, then tightly packed into the silo and heavily weighted. The silage produced was brownish in color, of pleasant odor, and was very readily eaten by the cattle. Carbone (5) states that this "Crema" silage is more palatable than A. I. V. silage.

#### FALAVIGNA METHOD

Falavigna (10) in Italy has advocated a method of making silage the essential feature of which seems to be ensiling at a temperature of 50° to 75° C. The silo is filled at such a rate that the forage reaches and maintains this temperature. The material is put in, one layer at a time, each layer being about 1.5 m. deep. Only when the temperature has reached 50° to 75° is another layer added. During the filling, salt is added at the rate of 2.8 pounds per ton. When the filling is completed, the forage is weighted down with stones, earth, or other loading material equivalent to 500 to 1,000 kg. per square meter. It is claimed that under these conditions lactic acid is the main product of fermentation, and butyric acid organisms are excluded by the high temperature.

A temporary silo, made of snow fencing and lined with Sisalcraft paper, was filled with unchopped alfalfa by this method. The temperature during the first week was 50° to 62° C. The analyses are given in table 3. The silage was low in acidity and conspicuously so in carotene, rather dark in color, and of a somewhat disagreeable odor. The palatability was only fair. There was considerable spoilage around the outside of the silo.

#### MIXED SILAGES

Mixed silages consisting of alfalfa plus various grasses were put up. The results are given in tables 1 and 2. The silages were only fair, and it would seem advisable to add molasses at about 30 pounds per ton in the preparation of these silages, as was done in the case of the pea vine-alfalfa silage.

The oat-pea bottle silage was put up while the forage was green and succulent, as can be seen from the dry-matter content (table 1), and made a product of very high carotene content.



Soybean silage is seen (table 2) to require more molasses than alfalfa silage. One hundred pounds per ton seemed to be satisfactory in both barrel and silo experiments.

### SUMMARY

Over a hundred lots of silage put up in many different ways over a period of several years have been compared as to appearance, odor, preservation, pH value, and carotene content.

Methods considered for the preservation of legume silages included, besides the standard A. I. V., molasses, and phosphoric acid methods, the use of such preservatives as whey powder, soured-whey concentrates, corn meal, sulfamic acid, soured molasses, sodium chloride and phosphorus pentasulfide. Of the latter, whey powder, soured-whey concentrate and corn meal gave the best results.

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# THE REDUCING-SUBSTANCE AND PHENOLIC-COMPOUND CONTENT OF THE POTATO TUBER IN RELATION TO DISCOLORATION AFTER COOKING <sup>1</sup>

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## INTRODUCTION

In previous publications (12, 8)<sup>2</sup> evidence was presented as to the relative instability of protein in potatoes which darken after boiling. It was found, moreover, (9)<sup>3</sup> that tyrosinase activity is considerably greater in tubers which discolor than in those which remain white after cooking. The presence of an activator or of activators of the oxidation of tyrosine in such abnormal potatoes was discovered. Although concentrated tuber extracts gave strong tests for the presence of dihydroxyphenylalanine or other carriers of the catechol structure, it was not possible to characterize any such compound. There was reason for supposing that failure in the available supply of one or more mineral elements was responsible for the metabolic irregularities mentioned above. However, a somewhat extensive examination (11) of the mineral content of tubers did not show a correlation between mineral deficiencies and blackening after cooking.

Szent-Györgyi (10) has attributed to quinones derived from the catechol structure the capacity to combine with proteins and amino acids to form brown to black pigments, called melanoids. This author supposes that the related oxidizing enzyme, polyphenolase, is rendered inactive in the normal cell and that its activity is released by injury, such as mechanical rupture.

On the other hand, Kertesz (3) found that lack of darkening in the Sunbeam variety of peach on exposure of its sliced tissue to the air is not due either to absence or to low activity of oxidizing enzymes. This peculiar difference of Sunbeam from other varieties was found to depend upon deficiency of catechol-tannin compounds. Reference is made by this author to the work of Nightingale, Addoms, and Blake (5), which showed that Elberta peaches produced on a low nitrogen supply were much higher in tannin than those from "high nitrogen trees."

## EXPERIMENTAL METHODS AND RESULTS

The work reported herein was directed toward more extensive determination of some of the reducing properties of tuber tissue than had been made in the preliminary examination (11, 12). It appeared desirable also, in this connection, to make a somewhat extended com-

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<sup>2</sup> Italic numbers in parentheses refer to Literature Cited, p. 358.

<sup>3</sup> ROSS, A. FRANK. THE RELATION OF CERTAIN NITROGENOUS CONSTITUENTS AND ENZYMES OF THE POTATO TUBER TO BLACKENING AFTER COOKING. 1937. [Doctor's thesis, Wis. Univ.]

parison of the catechol content, or its equivalent, and the discoloration record of potatoes.

In the preparatory phases of this investigation relatively unrefined procedure was applied to the measurement of discoloration. The half tubers produced by longitudinal bisection after paring in an abrasive machine were dropped into cold water and boiled until they were readily penetrated by a spatula. They were then removed to a sheet of filter paper, flat face upward, and examined after about half an hour under a fluorescent lamp in a compartment painted black internally. This appears to be a crude method of measurement, but attempts to make use of direct, ocular reflectometer readings on the mashed tissue, packed to a flat surface, were unsatisfactory. The results obtained by this latter method did not give the differences found by direct observation of both localized and diffused blackening of the un mashed potatoes.

#### THE TOTAL REDUCING CAPACITY OF TUBER TISSUE

##### OLD AND NEW POTATOES

The determination of the total reducing capacity of tuber tissue was the most general of several determinations made for comparison with the contemporaneous color record of boiled potatoes. In most of the work the tubers were pared before boiling, but in the earlier experiments they were boiled in the skins and peeled before inspection for discoloration. It was found that the flesh of cooked sections of unpared tubers, examined when resting upon the epidermis on filter paper, appeared about one grade darker—e. g., dark instead of medium gray—than they did after the epidermis was removed. The total content of reducing substances was determined by the method of Pett (6). In this procedure a tissue sample of appropriate weight is ground in a 3.0-percent solution of sulfosalicylic acid, filtered rapidly on a Büchner funnel, washed twice with small volumes of water, and titrated with 0.01 molar potassium iodate, using starch as an indicator.

A preliminary examination was made of 14 samples, covering 6 varieties of potatoes. These were produced mostly in Wisconsin and had been in storage at about 4.5° C. (40° F.) from November 1938 to July 1939. The boiling tests gave results essentially similar to those obtained in December. Values ranging from the equivalent of 15.7 to 21.4 mg. of ascorbic acid per 100 gm. of unpared tuber tissue were obtained, the order of reducing capacity bearing no relation to the degree of discoloration. The ascorbic acid content of these samples was also determined by the current procedure of titrating with dichlorophenol-indophenol. This specific reducing factor ranged in content from 6.3 to 10.8 mg. per 100 gm. of tissue, likewise irrespective of the variation of color from white to medium gray after boiling.

New potatoes of the Triumph and White Rose varieties purchased on the market at this time as received from California and Oregon each contained, within the limits of error, 23 mg. of ascorbic acid and 32 milligram-equivalents of this factor as total reducing capacity. Only a small proportion of the discrepancy between old and new potatoes as to content of ascorbic acid could be accounted for by reversible oxidation, because the stored tubers gave increases of only 1 to 3 mg. on reducing with hydrogen sulfide and removing the residue

of this gas in a current of nitrogen. Due allowance was made for 0.25 cc. of dye solution consumed as the average of several blank determinations of the reduction treatment.

While the results indicate that freshly harvested potatoes may be protected against discoloration, at least in part, by higher levels of ascorbic acid, it does not follow that this abnormality develops in proportion to the destruction of ascorbic acid during fall storage. The potatoes stored for 9 months therefore contained some 60 percent less ascorbic acid and 40 percent less total reducing substances than did the new potatoes examined. It has previously been reported (12) that blackening of cooked potatoes is not associated with immaturity of the tubers. The evidence for difference in total reducing power between old and new potatoes as an explanation of discoloration after cooking does not seem to be convincing.

In addition to several samples of Rural New Yorker, a few samples of other varieties were examined for their reducing capacity from 2 to 8 weeks after they were harvested. The tubers were placed in the cold storeroom in the first week of October about 1 week after harvest. Attempts were made to apply selective methods for determining glutathione and ascorbic acid, but these were abandoned in favor of the iodate titration of Pett (6). The procedures showed signs of promise, however. In the method of Langou and Marenzi (4) for joint determination of ascorbic acid and glutathione by means of Folin's phosphotungstic acid reagent, using cystine for the standard, the greenish color of the unknown as compared with the blue standard renders use of the Duboseq colorimeter infeasible. If proper filters were available, this method might be adapted to the use of the photoelectric colorimeter. The method of Binet and Weller (2) for determining glutathione, involving precipitation with cadmium lactate at a specific pH value and titration of the precipitate with potassium iodate, showed some promise of reliability. However, rather than determine individual constituents of questionable significance, it was decided to measure the total reducing level of tissue extracts. The results of this examination appear in table 1.

TABLE 1.—*Relation between the total reducing value and the discoloration after cooking of potato-tubers of 4 varieties taken from cold storage 2 to 4 weeks after harvesting*

Sample No.	Variety	Discoloration after cooking <sup>1</sup>	Original reducing values of raw tissue <sup>2</sup>	Reducing values after reduction by zinc <sup>2</sup>
1	Chippewa	O	23.6	27.6
2		O	28.0	28.0
3		O	27.8	28.2
4	Rural New Yorker	O	29.4	30.4
5		O	24.8	23.6
6		?	21.4	
7	Sebago	O	23.7	23.3
8		O	25.5	28.0
9	Cobbler	++	21.6	24.0
10		++	26.0	25.0
11		++	18.8	26.0
12		++	17.0	19.0
13	Rural New Yorker	++	20.5	21.2
14		++	17.4	18.0
15		++	20.5	

<sup>1</sup> O = white; ? = light gray; + = medium gray; ++ = dark gray.

<sup>2</sup> Milligram-equivalents of ascorbic acid per 100 gm. of tissue.

In view of the additional data indicative of general absence of reversibly oxidized forms of reducing substances, the determinations on this fraction were discontinued. The direct reducing values for the discolored Rural New Yorker samples were about 40 percent less than for those which were normal after cooking. However, not only the white-cooking Chippewa and Sebago samples but also the discoloring Cobblers showed values falling within the range covered by the better Rural New Yorker samples. Therefore, the results do not support unqualifiedly the concept of protection by reducing substances against discoloration.

As a further test of relationship between total reducing substances and discoloration after cooking, the records of a few samples which showed the common phenomenon of localized discoloration in the stem end of the tubers were examined. As in the preceding determinations, a 5.0-gm. sample was taken from the mixed tissue of several tubers. A record of the results is given in table 2. It will be observed that the data vary extremely from sample to sample. The small difference in some cases in the reducing value of the two ends indicates that much more extensive data would be required to disclose a definite trend, if such exists.

TABLE 2.—*Relation between the total reducing value and the discoloration after cooking of parts of Rural New Yorker potato tubers taken from cold storage 3 weeks after harvesting*<sup>1</sup>

Sample No.	Tuber end	Discoloration after cooking <sup>1</sup>	Reducing value of raw tissue
1	(Stem.....)	?	21.4
	(Bud.....)	0	18.2
2	(Stem.....)	++	19.0
	(Bud.....)	+	8.4
3	(Stem.....)	++	17.2
	(Bud.....)	+	23.5
4	(Stem.....)	++	16.6
	(Bud.....)	+	14.2

<sup>1</sup> See footnotes to table 1.

The search for relationship between total reducing capacity of the tissue and discoloration after cooking was extended to a few more samples with the results shown in table 3. These data do not show a greater content of reducing substances in the samples which are less subject to discoloration. Their import is supported by the results from a series of Rural New Yorker plantings conducted in cylinders of soil sunk in the field for purposes of control over the supplies of water and mineral nutrients. The crops cooked white throughout but showed a range of from 5.8 to 19.3 mg. of ascorbic acid equivalent as the total reducing capacity. We are thus led to conclude that there is no direct relation between total reducing substances in the raw tuber and whiteness after boiling.

TABLE 3.—*Relation between the total reducing value and the discoloration after cooking of potato tubers of 2 varieties taken from cold storage 5 to 6 weeks after harvesting*<sup>1</sup>

Sample No.	Variety	Discoloration after cooking	Reducing value of raw tissue
1	Rural New Yorker	O	18.3
2		?	17.4
3		?	13.0
4		+	18.3
5	Green Mountain	+	19.0
6		+++	15.0
7		+++	18.6
8	Rural New Yorker	+++	13.7
9		+++	17.6
10		+++	14.3

<sup>1</sup> See footnotes to table 1.

## THE CATECHOL EQUIVALENT OF POTATOES

A series of samples of the Rural New Yorker variety, produced at various locations in Wisconsin was placed at the writers' disposal by Prof. G. H. Rieman of the Wisconsin Agricultural Experiment Station. In view of the activating effect of catechol upon the tyrosinase system (7, pp. 467-471) and because of its localization in the cortical region, these samples were subjected to determination of the catechol grouping and to other examination in both the pared and the unpared states. Individual tubers were sectioned longitudinally to provide composite samples. Some of the sections were then analyzed and compared with the boiling record of corresponding sections. The same treatment was applied to other sections of each sample after they had been pared in a small machine of the abrasive type. For the purpose of comparison, the total reducing capacity was determined in the usual manner. The catechol equivalent was determined by treating a portion of the tuber extract according to the procedure of Arnow (1). A 2-cc. sample was placed in a colorimeter tube and 2-cc. portions of 0.5 N HCl, 10-percent nitrite-molybdate solution, and 1.0 N NaOH were added successively with shaking. The solution was made to a volume of 10 cc. with water and examined in the Evelyn photoelectric colorimeter with the use of a color filter effective at 5,400 Å. The concentration was determined by comparison with a curve prepared by use of standard solutions of catechol. Data from these several determinations are assembled in table 4.

TABLE 4.—*Influence of paring upon the discoloration after cooking, the reducing value, and the catechol content of Rural New Yorker potato tubers grown in different localities and taken from cold storage 8 weeks after harvesting*

Sample No.	Where grown	Discoloration after cooking <sup>1</sup>		Reducing value of raw tissue <sup>2</sup>		Catechol equivalent <sup>3</sup>	
		Pared	Unpared	Pared	Unpared	Pared	Unpared
1	Delavan	O	O	19.2	12.5	2.0	5.8
2	Rice Lake	Od	OD	22.5	24.0	2.7	3.9
3	Starks	++	+++d	10.6	18.5	2.4	6.6
4	Antigo	+++d	+++d	17.4	17.7	3.5	5.2
5	Sturgeon Bay	+++d	+++d	12.4	16.5	3.4	7.5
7	Rockfield	++	+++	17.1	14.9	3.8	8.2

<sup>1</sup> O = white; ++ = medium gray; +++ = dark gray; d = mildly discolored cortex; D = seriously discolored cortex.<sup>2</sup> Milligram-equivalents of ascorbic acid per 100 gm. of tissue.<sup>3</sup> Milligram per 100 gm. of tissue.



The most prominent feature of the data is the rather consistent increase of catechol equivalent in the pared tuber in relation to blackening after cooking. More data are presented in tables 5 and 6 which support this correlation substantially. It further appears that catechol, or compounds reacting like it, is relatively concentrated in the epidermis and underlying tissues but that discoloration is not directly related to this localization. More data would be required, however, to justify emphasis upon this issue.

Further evidence was sought by comparing tissue from a longitudinal V-shaped section with a portion from a similar section of the same tuber which constituted a thin peel. A sample of Cobbler which cooked light gray contained 6.0 mg. of catechol equivalent in 100 gm. of the total tissue, compared with 13.8 mg. in the peel. Using the plug method, to be described presently, the corresponding values for a Rural New Yorker sample which cooked medium gray were 5.4 and 9.5 mg., respectively.

Additional results which show general correlation between the catechol equivalent and discoloration during cooking, but a partly inverse relation in regard to the total reducing substances, are presented in table 5. The samples here considered were produced from a discoloring seed stock with different fertilizer treatments. As usual, the discoloration bore no consistent relation to any formula of the applied fertilizer.

TABLE 5.—*Relation between the total reducing value and the discoloration after cooking of Rural New Yorker potato tubers taken from cold storage 11 weeks after harvesting*<sup>1</sup>

Sample No.	Discoloration after cooking	Reducing value of raw tissue	Catechol equivalent
1	O	14.5	4.6
2	++	14.9	4.8
3	++	14.6	5.4
4	++	13.3	6.2
5	+++D	13.3	5.8
6	+++D	11.9	6.2
7	++++	10.5	7.3

<sup>1</sup> See footnotes to table 4.

#### THE PLUG METHOD OF COMPARING DISCOLORATION WITH REDUCING-SUBSTANCES CONTENT

In view of the concentration of catechol, or compounds reacting as such, in the epidermis or underlying cortical tissue, it became apparent that the tubers should be so sampled as to avoid the effect upon total tissue composition of decrease in proportion of surface as the tubers increased in size. This problem was solved by taking plug or partial core samples from each tuber and using a definite length of such core from the epidermis inward. Five tubers which had been found to discolor seriously by boiling a section of each were pierced longitudinally from the stem end by a No. 8 cork borer. Rapidly, in order to minimize oxidative changes, portions about 1.0 cm. in length were cut from the stem end of these cores to give plugs which bore the epidermis. The plugs were trimmed on their inner ends to give a composite sample weighing 10 gm. This sample was promptly covered

with a 3-percent solution of sulfosalicylic acid and thoroughly crushed in a mortar. It was found necessary to use small-scale filtering equipment (4.25-cm. Büchner funnel and 50-cc. filtering flask) in order to obtain satisfactory agreement of results from duplicate samples. The determinations of total reducing capacity and catechol equivalent were made in the usual manner, but the determination of ascorbic acid was modified by use of an Evelyn photoelectric colorimeter. The results from different varieties and strains thereof as produced on clay loam at Antigo and sandy loam at Hancock in 1939 are shown in table 6. It should be observed that, with the approach of the spring break in dormancy, most varieties could be expected to turn somewhat gray after cooking.

TABLE 6.—*Relation between the discoloration after cooking and certain reducing properties of the tuber tissue of different varieties and strains of potatoes, produced at different locations in 1939, and taken from cold storage 12 to 14 weeks after harvesting*<sup>1</sup>

Sample No.	Variety and strain	Where grown	Discoloration after cooking	Reducing value of raw tissue	Ascorbic acid content	Catechol equivalent
1	Chippewa, A. ....	Antigo	O	12.2	7.6	3.3
2			O	12.6	8.5	3.5
3			O	13.3	8.5	3.5
4			O	13.7	7.8	4.2
5			+	14.3	10.6	3.2
6	Cobbler, A. ....		+	15.2	12.6	3.6
7			++	16.3	12.9	3.6
8			++	16.3	13.5	3.4
9			++	13.8	9.6	5.4
10			++	14.8	11.2	5.3
11	Rural New Yorker, A. ....		++	15.4	11.4	5.4
12			?	15.7	.....	3.6
13			++	14.9	.....	4.8
14			++	17.9	12.6	5.1
15			?d	18.4	14.9	4.9
16	Rural New Yorker, B. ....		?d	17.7	14.3	4.1
17			+	13.9	10.7	6.0
18			++d	16.8	15.2	5.9
19			++	18.1	16.3	4.5
20			?	17.2	10.1	3.9
21	Rural New Yorker, C. ....		++	13.8	8.6	5.0
22			++	13.5	6.1	5.8
23			++	14.6	8.5	5.4
24			++d	10.8	.....	5.5
25			++	13.0	.....	4.5
26	Rural New Yorker, A. ....	Hancock	++	12.7	6.1	5.4
27			++	5.9	.....	5.8
28			++	12.5	6.6	6.8
29			++D	9.5	.....	6.3

<sup>1</sup> See footnotes to table 4.

Practically all of the Rural New Yorker samples and all of the Chippewa samples from Antigo were produced with different fertilizer treatments. These included graded levels of potassium supply and the supplementary use of carriers of boron, copper, manganese, and zinc. There is no evidence of correlation between the degree of discoloration and the plane of supply of mineral nutrients. The different strains of Rural New Yorkers are tabulated separately because in the fall boiling test they had given increasing discoloration in the order in which they are recorded. At Hancock the crop came from unfertilized divider rows. This region has the most serious record of discoloring potatoes over a 5-year period in the writers'

experience. Ascorbic acid determinations were purposely omitted in a few cases because of lack of correlation between tuber content of this factor and the record of discoloration in a large number of examinations.

The data show no higher content of total reducing substances in the Chippewa, which cooked white, than in some of the most seriously discoloring samples of Cobbler and Rural New Yorker. Moreover, the predominantly high content of this tissue fraction in the latter varieties as produced at Antigo in contrast to the Rural New Yorker samples from Hancock does not indicate that the general reductive capacity of the tuber tissue has a protective function against pigmentation after cooking.

The ascorbic acid content does not seem to have varied at the time of examination in a manner related to blackening after boiling. In fact, its values show much the same trend as those for total reducing substances. The values for Chippewa are as low as those for some of the most discoloring Rural New Yorker samples.

There appears to be, however, a considerable degree of correlation between the values for catechol and the degree of discoloration. The average value of the catechol equivalent for the different groups of samples is as follows: Chippewa 3.6, Cobbler 4.3, Antigo Rural New Yorker 4.9, Hancock Rural New Yorker 5.7. This record is in accord with the discoloration findings for these varieties and locations over a 5-year period of investigation. In contrast, the average values for ascorbic acid and total reducing capacity are essentially equal for the Cobbler and Rural New Yorker varieties from Antigo and decline markedly in the Hancock crop. It should be noted, however, that the white-cooking Chippewa contained as much catechol equivalent as some of the discoloring Cobblers. The Rural New Yorker shows the most definite correlation between the level of catechol content and the degree of blackening after cooking. Since the tabulated data were obtained, the following results have been secured from the 1940 crop held in cold storage until December or January: Rural New Yorker cooking white, 3.2 and 3.8; Chippewa cooking white, 3.8, light gray, 4.9; Rieman's crosses cooking medium gray, with seriously discolored cortex, 6.5, 6.6, and 8.7.

The present results emphasize the relative abundance of compounds containing the catechol grouping in potatoes that discolor when boiled. Because of the limitations already noted with respect to the evaluation of discoloration, one can hardly expect close correlation between this function and compositional factors. It can be stated with certainty that the compound causing discoloration is related to orthodihydroxyphenol because, in contrast with the pink color of the nitroso derivative here obtained, parahydroxyphenol yields an unstable derivative which turns brown rather promptly, and metahydroxyphenol does not produce a color. This makes it possible that the presence of orthoquinone contributes to pigment formation in accordance with the concept of Szent-Györgyi (10, p. 68.)

Spectrophotometric absorption curves of the fraction precipitated by basic lead from alcoholic extracts of discoloring potatoes were found to have nearly the same characteristics as those of catechol and dihydroxyphenylalanine (dopa). The writers found, as had Arnow (1) in attempting to determine tyrosine and dihydroxyphenylalanine

in a common solution, that the characteristic wave lengths and the breadths of the curve maxima are too similar to permit use of this property for identification. Therefore, we are attempting to isolate derivatives of the compound or compounds involved, for purposes of characterization and identification.

### SUMMARY

Earlier work of this laboratory disclosing the presence of an activator of tyrosinase in potatoes which blacken after cooking has been reviewed. Extracts of such disclosing tubers were found to contain an orthodihydroxyphenolic compound, or compounds, resistant to isolation.

Tubers of several varieties from the Wisconsin crop which had been stored at about 4.5° C. for 9 months were about 60 percent lower in ascorbic acid content and 40 percent lower in total reducing substances than Triumph and White Rose potatoes recently shipped to local markets from the West Coast. These were found to contain little reversibly oxidized ascorbic acid.

After cold storage for 2 to 4 weeks after harvest, Rural New Yorker potatoes which discolored contained about 40 percent less total reducing substances (titratable by  $\text{KIO}_3$ ) than did those which cooked normally white. In comparison with the composition of other varieties from the same storage the results do not indicate a protective effect of the total reducing function against the blackening response. Examination for the accumulation of reversible products of oxidation gave generally negative results.

A limited comparison of the total reducing values of tissue from the stem and bud ends of tubers which had been indexed for serious discoloration did not indicate that localized darkening commonly observed in the former region could be ascribed to deficiency of reducing substances.

A comparison of mildly with seriously discoloring Rural New Yorker samples after cold storage for 5 to 6 weeks showed no difference in the range of reducing capacity. An examination of ascorbic acid content in crops of Rural New Yorker produced under different cultural conditions showed that low levels of this constituent were not related to blackening after cooking.

Rural New Yorker potatoes in cold storage for 8 weeks after harvest showed marked concentration in the epidermis and cortex (peel) of a substance or substances giving the catechol reaction. The total reducing substances were not distributed in this manner. After storage for 11 weeks this variety was found to contain amounts of catechol which correlated well with the degree of blackening after boiling, while a somewhat inverse relation existed with regard to total reducing substances.

A method of sampling the tuber tissue (core taking) was adopted to limit the variation between epidermis-cortex and the parenchyma tissue. About 29 samples of three varieties, including the Rural New Yorker from two regions, were examined by this procedure 12 to 14 weeks after they had been placed in cold storage. Higher content of total reducing substances was not correlated with freedom from discoloration after cooking, nor was higher ascorbic acid content.

The content of compounds reacting like, and expressed as the equivalent of, catechol increased from sample to sample somewhat in proportion to blackening after boiling.

In view of the inapplicability of spectrophotometric absorption curves for identifying the orthodihydroxy compound, or compounds, apparently associated with the blackening of boiled potatoes, methods of isolation, and chemical characterization are being applied.

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# PHYSIOLOGIC STUDIES OF RHIZOBIUM MELILOTI, WITH SPECIAL REFERENCE TO THE EFFECTIVENESS OF STRAINS ISOLATED IN KANSAS<sup>1</sup>

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## INTRODUCTION

Investigations conducted at the Kansas Agricultural Experiment Station have revealed remarkable losses in the nitrogen content of Kansas soils under cultivation. Certain data presented by Swanson and Latshaw<sup>2</sup> indicate that losses in the soil's store of nitrogen may take place even under continuous cropping to alfalfa. Since alfalfa is generally recognized as one of the most efficient nitrogen-gathering members of the Leguminosae, the data presented by Swanson and Latshaw suggest either the absence of adequate numbers of the alfalfa-sweetclover group (*Rhizobium meliloti* Dangeard) of rhizobia in Kansas soils or the presence of relatively low nitrogen-fixing strains. In view of the role that alfalfa and sweetclover play in the soil improvement program in Kansas it seemed worth while to obtain information relative to the distribution and nitrogen-fixing efficiency of Kansas strains of rhizobia capable of infecting these two crops.

A preliminary survey in the vicinity of Manhattan indicated an abundant soil flora capable of producing nodules on alfalfa and sweetclover, provided soil conditions were favorable. This apparent abundance of *Rhizobium meliloti* suggested that if the rhizobia were in any way at fault in the nitrogen-fixing phenomenon, it was probably due to the presence of strains with relatively low nitrogen-fixing capacity.

## METHODS AND MATERIALS

### ISOLATION OF CULTURES

Nodules were obtained from cultivated alfalfa (*Medicago sativa* L.) plants and from sweetclover (*Melilotus alba* Desr.) plants found along roadsides. Selected nodules were washed in water, immersed approximately 5 minutes in a 1-500 solution of bichloride of mercury, and rinsed in sterile water. Poured plates were then prepared with nitrate-mannitol agar or the medium proposed by Albrecht and McCalla.<sup>3</sup> After incubation for several days at 28° to 30° C., characteristic colonies were selected, tested for purity, and the cultures were kept on the Albrecht-McCalla medium.

### TESTING THE INFECTIVENESS AND EFFICIENCY OF CULTURES

Six hundred grams of washed general-run Ottawa sand, mixed with a little calcium carbonate, was introduced into 65 by 500 mm. pyrex test tubes. One hundred thirty-five milliliters of modified Crone's

<sup>1</sup> Received for publication March 18, 1940. Abstract of an unpublished thesis submitted by the senior author to the faculty of Kansas State College of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of doctor of philosophy. Contribution No. 184, Department of Bacteriology, Kansas Agricultural Experiment Station.

<sup>2</sup> SWANSON, C. O., and LATSHAW, W. L. EFFECT OF ALFALFA ON THE FERTILITY ELEMENTS OF THE SOIL IN COMPARISON WITH GRAIN CROPS. *Soil Sci.* 8: 1-39, illus. 1919.

<sup>3</sup> ALBRECHT, WILLIAM A., and MCCALLA, T. M. A NEW CULTURE MEDIUM FOR RHIZOBIA. *Jour. Bact.* 34: 455-457. 1937.

solution,<sup>4</sup> or 15 milliliters less than the amount retained against gravity by the sand, was added. This volume of liquid plus the 15 milliliters of inoculum gave satisfactory moisture conditions. The tubes were plugged with cotton and sterilized at 20 pounds pressure for 1½ hours.

In experiment No. 7 milk bottles were substituted for the pyrex tubes, and approximately 50 percent longer was required to obtain an equal amount of growth; otherwise the results were apparently comparable. Decreased growth in milk bottles was probably due to less adequate lighting.

Alfalfa seeds were shaken for 15 minutes in a 1-500 solution of bichloride of mercury, washed six or more times in sterile distilled water, and germinated on nutrient-agar plates at 28° to 30° C. Eight seedlings were introduced into each tube and the sand medium was inoculated with 15 milliliters of a heavy suspension of cells prepared from young agar slant cultures.

The tubes were then taken to the greenhouse where the plants were allowed to grow for about 7 weeks. If the greenhouse temperature could not be kept low enough to permit good growth of alfalfa, the cultures were transferred to a partially shaded position outdoors where the tubes were buried in the soil to the depth of the sand, and protected from rain by inverting a small tin can over each tube. The volume of liquid held by the 600 gm. of sand was adequate to supply the needs of the plants during the entire growing period; hence, it was not necessary to open the tubes following inoculation.

The following facts would indicate that the conditions within this enclosed system were satisfactory for the object in view: (1) Vigorous growth of the alfalfa plants invariably occurred when inoculated with an effective culture; (2) in no instance did a single plant fail to show one or more nodules when an infective culture was employed as the inoculum; (3) in no instance did an uninoculated plant develop a nodule; (4) the number of nodules developed per plant averaged in excess of 20 in case of highly infective strains; (5) plants invariably made poor growth and exhibited typical nitrogen-hunger symptoms unless inoculated.

Burton and Wilson<sup>5</sup> obtained evidence that the fixation of nitrogen by a strain of *Rhizobium meliloti* may vary with the variety of host plant. Since about 90 percent of the alfalfa grown in Kansas is Kansas Common (*Medicago sativa*), this variety was employed in all tests.

After a growing period of approximately 7 weeks, the plants and sand were emptied onto a sieve from which the sand could be washed readily. The number of plants and nodules in each tube was recorded, and all plant material was oven-dried at 110° C. and analyzed for total nitrogen by the Gunning method.<sup>6</sup> In view of the large amount of sand per tube no attempt was made to recover the nitrogen that may have been excreted into the medium, and it is possible that some nitrogen may have been lost in this way.<sup>7</sup>

<sup>4</sup> FRED, EDWIN BROWN, BALDWIN, IRA LAWRENCE, and MCCOY, ELIZABETH. ROOT NODULE BACTERIA AND LEGUMINOUS PLANTS. Wis. Univ. Studies Sci. 5: 343 pp., illus. 1932.

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<sup>6</sup> ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS. OFFICIAL AND TENTATIVE METHODS OF ANALYSIS . . . Compiled by the committee on editing methods of analysis. Ed. 4, 710 pp., illus. Washington, D. C. 1935.

<sup>7</sup> VIRTANEN, A. I. ASSOCIATED GROWTH OF LEGUMES AND NONLEGUMES. 4th Internatl. Grassland Cong., Aberystwyth, Gr. Brit., Rpt., pp. 78-88, illus. 1937.

## EXPERIMENTAL DATA AND DISCUSSION

In measuring the physiological efficiency of a series of unknowns it is obviously desirable to have some standard with which to make comparisons. There are no standard methods of testing the efficiency or ascertaining the quantities of nitrogen that must be fixed by a given culture of rhizobia before it can be rated as an efficient, or "good," strain. In fact, there are few records of performance available. Under such conditions the most logical procedure appeared to be to obtain from other laboratories cultures rated as efficient and make use of certain of these for comparative purposes. Accordingly, a number of strains of *Rhizobium meliloti* were obtained from research and commercial laboratories, two of the most efficient were selected, and one or both were included in every series of tests conducted. Both strains gave consistently good nodulation, good growth of plant, and somewhat better fixation of nitrogen than the average of locally isolated cultures; hence, served satisfactorily for comparative purposes. In subsequent discussions these two strains, No. 107 from the University of Wisconsin, and A from the Urbana (Ill.) Laboratories, will arbitrarily be regarded as efficient strains, though the limitations in their use as a basis for grading the relative merits of other strains should be kept in mind.

Another fundamental question involved in comparing the relative merits of a series of unknowns is the degree of accuracy of the data upon which the comparison is based. The facilities and time available for conducting such tests were so limited as to restrict the adequate replication of tests for statistical treatment to a very small number of strains. It was believed that a limited study of a large number of strains would give more information relative to the efficiency of the local rhizobia than would a more concentrated study of a few. Consequently each culture isolated was tested once, and when a given strain showed evidence of marked nitrogen-fixing inefficiency it was retested. The retesting of only the apparently inefficient strains was based upon the assumption that the chances for an error in technique being responsible for a combination of good growth and marked accumulation of combined nitrogen, in the absence of fixation by the culture in question, were extremely small. On the other hand, only slight alterations in the environmental conditions might have such an effect upon either of the symbionts as to prohibit nitrogen fixation. This assumption seemed justifiable in view of the fact that the pabulum contained only small quantities of combined nitrogen and all extraneous nitrogen-fixing organisms were eliminated.

In order to secure some concrete information as to the variability in growth, modulation, and nitrogen fixation to be expected in replicated tests of individual strains (a) 12 different cultures were replicated 6 times in a single experiment, and (b) 1 or both of the 2 cultures chosen for comparison were included in every experiment. In (a) the experimental conditions for each of the individual tests of the 12 strains were as nearly identical as it was possible to make them, whereas in (b) the 2 strains were subjected to as wide variations as were encountered in the study. It is believed, therefore, that a consideration of the data presented in tables 1 and 2 will give a fair indication of the latitude in growth, modulation, and nitrogen fixation resulting from the experimental error under the experimental conditions employed.



TABLE 1.—*Nodulation, growth, and fixation of nitrogen in the presence of 12 different strains of Rhizobium meliloti*

Strain No.	Plants per culture	Nodules		Dry weight		Nitrogen fixed		Nitrogen content of plants	
		Per plant	Mean <sup>1</sup>	Per plant	Mean	Per plant	Mean <sup>1</sup>	Per group	Mean
	Number	Number	Number	Milli-grams	Milli-grams	Milli-grams	Milligrams	Percent	Percent
Control	6	0.0	-----	13	14	0.00	-----	1.38	1.35
	6	0		15		0		1.21	
	6	0		14		0		1.35	
A.	7	3.6	4.0±.398	18	19	.43	0.45±.011	3.31	3.36
	6	2.8		19		.46		3.30	
	6	4.0		19		.45		3.33	
	5	5.0		21		.48		3.41	
	6	4.7		18		.42		3.43	
4.	6	4.2	4.9±.438	18	18	.45	.45±.037	3.39	3.51
	6	4.8		16		.41		3.70	
	7	4.9		15		.36		3.48	
	6	4.0		18		.45		3.48	
	4	6.5		25		.58		3.51	
12-II	6	2.7	3.7±.160	17	17	.43	.43±.014	3.62	3.68
	6	4.2		16		.39		3.68	
	6	3.3		16		.42		3.79	
	6	2.5		18		.47		3.65	
	6	5.8		18		.46		3.65	
18-II	6	6.0	6.2±.329	18	17	.36	.36±.015	2.97	3.25
	6	5.7		17		.38		3.40	
	6	6.8		16		.33		3.39	
27-I	6	7.7	8.2±1.020	16	16	.38	.34±.023	3.55	3.30
	6	6.8		16		.34		3.27	
	6	10.2		16		.30		3.08	
32-III	8	5.0	8.0±1.340	17	17	.36	.35±.025	3.21	3.22
	6	12.7		19		.30		2.53	
	6	6.8		14		.31		3.62	
	6	9.0		19		.43		3.30	
	6	6.5		15		.34		3.45	
54-I	8	4.0	4.3±.304	15	16	.39	.36±.019	3.54	3.36
	6	5.3		16		.31		3.48	
	7	3.9		15		.36		3.49	
	6	3.7		18		.42		3.39	
	6	4.8		18		.34		2.88	
61-I	6	6.3	4.4±.576	19	19	.49	.47±.018	3.49	3.53
	6	4.2		20		.51		3.52	
	6	4.7		18		.47		3.31	
	6	3.2		16		.46		3.04	
	6	2.5		21		.51		3.40	
	6	5.5		17		.40		3.51	
63-I	7	2.6	3.8±.331	14	17	.32	.42±.027	3.39	3.48
	6	3.3		16		.46		3.90	
	6	4.0		16		.35		3.28	
	5	5.0		19		.48		3.59	
	6	4.2		19		.46		3.34	
	6	3.8		18		.44		3.39	
101	6	4.5	4.2±.325	17	19	.48	.47±.021	3.90	3.47
	6	4.5		20		.49		3.32	
	6	3.2		18		.41		3.31	
	6	4.5		21		.51		3.33	
107-I	6	3.5	3.7±.229	18	19	.47	.52±.016	3.71	3.82
	6	4.2		18		.56		4.20	
	6	2.7		23		.54		3.23	
	6	3.8		18		.47		3.73	
	6	4.2		19		.51		3.66	
	6	3.8		17		.54		4.37	
110	6	4.5	4.3±.425	16	18	.43	.45±.013	3.85	3.50
	6	3.7		17		.41		3.46	
	6	3.2		17		.46		3.76	
	6	5.7		20		.47		3.32	
	6	4.5		21		.48		3.13	

<sup>1</sup> Standard error of mean is used.

TABLE 2.—*Nodulation, growth, and fixation of nitrogen in the presence of strains A and 107-I of Rhizobium meliloti*

STRAIN A <sup>1</sup>					
Experiment No.	Plants per culture	Nodules per plant	Dry weight per plant	Nitrogen fixed per plant	Nitrogen in plants
	Number	Number	Milligrams	Milligrams	Percent
1.....	7	3.6	18	0.43	3.31
1.....	6	2.8	19	.46	3.30
1.....	6	4.0	19	.45	3.33
1.....	5	5.0	21	.48	3.41
1.....	6	4.7	18	.42	3.43
3.....	7	3.7	20	.54	3.39
4.....	7	5.1	13	.32	3.53
5.....	6	5.5	16	.41	4.01
6.....	6	8.0	22	.52	3.08
7.....	5	5.2	24	.48	2.83
8.....	5	7.2	23	.47	3.08
9.....	5	9.6	42	.67	2.20
10.....	6	8.0	23	.49	2.82
Mean.....	6	5.6	21	0.47	3.21
Standard error of mean.....		±0.539	±1.92	±0.024	±0.120

STRAIN 107-I <sup>2</sup>					
Experiment No.	Plants per culture	Nodules per plant	Dry weight per plant	Nitrogen fixed per plant	Nitrogen in plants
	Number	Number	Milligrams	Milligrams	Percent
1.....	6	3.5	18	0.47	3.41
1.....	6	4.2	18	.56	4.20
1.....	6	2.7	23	.54	3.23
1.....	6	3.8	18	.47	3.73
1.....	6	4.2	19	.51	3.66
1.....	6	3.8	17	.54	4.37
2.....	7	5.0	21	.54	2.99
3.....	7	5.6	15	.33	3.11
4.....	6	5.2	13	.38	4.17
5.....	6	3.8	12	.27	4.19
6.....	7	4.9	17	.46	3.59
7.....	6	7.3	22	.38	2.50
8.....	7	10.3	21	.49	3.18
9.....	5	9.6	33	.46	2.11
10.....	5	11.4	30	.69	2.82
Mean.....	6	5.7	20	0.47	3.42
Standard error of mean.....		±0.610	±1.466	±0.0266	±0.171

<sup>1</sup> Correlation coefficient between the number of nodules and nitrogen fixed = +0.553; significant at 5-per-cent level.

<sup>2</sup> Correlation coefficient between the number of nodules and nitrogen fixed = +0.262; not significant at 5-per-cent level.

Four of the cultures employed in experiment 1 (A, 107, 110, and 101) were received from other laboratories; the remaining eight were isolated locally. Cultures A and 107 were described as "good," 101 as "fair," and 110 as "poor." Preliminary tests had indicated that cultures 4, 18-II, 27-I, 32-III, and 54-I combined in varying degrees the characteristics of inferior nitrogen fixation with relatively high nodule production. On the other hand, cultures 12-II, 61-I, and 63-I had exhibited both good nitrogen fixation and low nodule production.

The data presented in table 1 indicate that four of the locally isolated cultures, i. e., 18-II, 27-I, 32-III, and 54-I, were inferior to the other four and to the four exogenous strains. Three of the inferior local cultures also produced a larger number of nodules. Cultures 4 and 110 apparently had undergone some alteration in nitrogen-fixing ability or perhaps should be classed as "erratic," a term that has been applied to strains exhibiting marked variability in nitrogen fixation.

These data indicate a more marked variability in the number of nodules produced by a given strain than in nitrogen fixed, the latter being rather uniform.

The data in table 1 were subjected to an analysis of variance to determine the significance of differences in the number of nodules formed and the quantities of nitrogen fixed by different strains of rhizobia. The results appear in table 3.

TABLE 3.—*Summary of analysis of variance in nitrogen fixation and nodule formation of strains of Rhizobium meliloti listed in table 1*

Item	Degrees of freedom	Sum of squares	Variance	F	5-percent level	1-percent level
Nitrogen fixation:						
Strains of rhizobia	11	0.164	0.0149	6.699	2.00	2.66
Error	46	.103	.0022			
Total	57	0.267				
Level of significance					0.031	0.041
Nodule formation:						
Strains of rhizobia	11	119.076	10.8251	14.080	2.00	2.66
Error	46	35.366	.7688			
Total	57	154.442				
Level of significance					0.568	0.759

If the value of *F* exceeds the values for the 5-percent and 1-percent levels, the odds are 19 to 1 and 99 to 1, respectively, that the variation is, correspondingly, significant and highly significant. If the differences in the mean values for nitrogen fixed and nodules formed, as recorded in table 1, exceed the levels of significance, then the chances are 19 to 1 and 99 to 1, respectively, that such differences are not due to chance.

In other words, the number of nodules formed by strains 18-II, 27-I, and 32-III are significantly higher than the number formed by the other strains, while the quantities of nitrogen fixed by these three strains and also by strains 54-I are significantly lower than for the other strains tested.

An application of the test for significance gave essentially the same results. It is evident from these data that variations in nodule formation and nitrogen fixation can be measured by the method employed in securing the data presented in table 1. It is also believed that these data give a good indication of the degree of variability in nitrogen fixed and nodules formed by a given culture when replicated under as nearly the same conditions as possible. Figure 1 illustrates the remarkable uniformity in growth observed in replicated tests. These data, however, give no indication of the variations that might attend such marked alterations in light and temperature as occurred in subsequent experiments as a result of inability to control these two factors adequately. In order to gain such information, cultures A and 107 were included in every series of tests. The data collected in this manner are recorded in table 2.

As would be expected, variations are to be noted in these data both as regards nitrogen fixed and nodules formed. With few exceptions, however, the values do not vary far from the means. Of the 28 individual tests here recorded of these 2 supposedly good strains, only 1 test fell within the range later to be designated as definitely non-efficient and only 5 fell below the general average for the 217 locally isolated cultures. In 1 test of each culture the quantities of nitrogen fixed were unusually high. Strain 107 exhibited a greater tendency

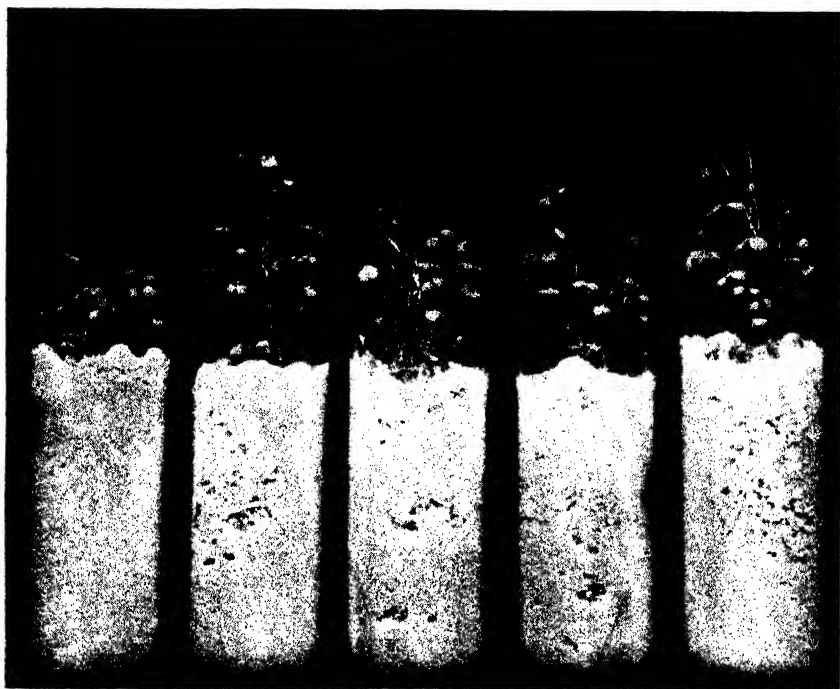


FIGURE 1.—The alfalfa plants in all five tubes were inoculated with the same strain (12-11) of *Rhizobium meliloti*.

to vary both in quantity of nitrogen fixed and in number of nodules formed than did culture A.

The positive correlation between the number of nodules formed and the quantities of nitrogen fixed, a correlation that is definitely significant in the case of strain A, is an interesting fact brought out by the data in table 2. This point will be referred to again.

Altogether 217 cultures of *Rhizobium meliloti* were isolated from alfalfa and sweetclover nodules collected within a radius of 50 miles of Manhattan, Kans. These were tested for their ability to form nodules and fix nitrogen in a series of eight experiments conducted during a period of slightly over a year. A presentation of the complete analytical data accumulated in connection with these tests does not seem essential. The data have been summarized and are presented in tables 4, 5, and 6.

TABLE 4.—*Nodulation, growth, and nitrogen fixation in the presence of locally isolated cultures of Rhizobium meliloti*

Experiment No.	Cultures tested	Average number of nodules per plant	Average weight per plant	Average nitrogen fixed per plant	Average nitrogen in plant	Correlation coefficient between nitrogen fixed and number of nodules <sup>1</sup>
	<i>Number</i>	<i>Number</i>	<i>Milligrams</i>	<i>Milligrams</i>	<i>Percent</i>	
2.....	45	9.4	23	0.41	2.73	<sup>2</sup> -0.393
3.....	21	6.5	19	.41	2.93	<sup>3</sup> -0.067
4.....	35	5.8	15	.38	3.73	<sup>3</sup> +0.045
5.....	19	5.1	15	.37	3.92	<sup>3</sup> +0.044
6.....	16	10.3	18	.34	2.77	<sup>4</sup> -0.613
7.....	36	7.8	21	.34	2.48	<sup>4</sup> -0.387
8.....	41	10.8	22	.45	2.94	<sup>2</sup> -0.492
9.....	4	16.3	12	.32	2.56	<sup>3</sup> +0.45
Mean.....		8.2	19	.39	3.01	

<sup>1</sup> The correlation coefficient between nitrogen fixed and nodules produced for the entire 217 cultures was -0.202, a highly significant value.

<sup>2</sup> Highly significant.

<sup>3</sup> Not significant.

<sup>4</sup> Significant.

TABLE 5.—*Classification of locally isolated cultures of Rhizobium meliloti according to nitrogen-fixing ability*

Experiment No.	Cultures tested	Cultures in indicated range of nitrogen fixation			
		<0.30 milligram	0.30-0.39 milligram	0.40-0.46 milligram	>0.46 milligram
	<i>Number</i>	<i>Number</i>	<i>Number</i>	<i>Number</i>	<i>Number</i>
2.....	45	11	13	8	13
3.....	21	3	8	3	7
4.....	35	5	18	6	6
5.....	19	4	7	7	1
6.....	16	3	10	2	1
7.....	36	12	12	1	11
8.....	41	5	7	10	19
9.....	4	2	0	1	1
Total.....	217	45	75	38	59
Proportion of total.....		<i>Percent</i> 20.7	<i>Percent</i> 34.6	<i>Percent</i> 17.5	<i>Percent</i> 27.2

TABLE 6.—*Relationship between number of nodules formed and nitrogen fixed by 217 cultures of Rhizobium meliloti*

Number of nodules per plant	Cultures	Average number of nodules	Average nitrogen fixed
	<i>Number</i>	<i>Number</i>	<i>Milligram</i>
Less than 5.....	48	3.9	0.41
Between 5 and 7.5.....	64	5.9	.40
Between 7.5 and 10.0.....	54	8.6	.41
Between 10.0 and 12.5.....	22	11.0	.43
Between 12.5 and 15.0.....	10	13.6	.38
Between 15.0 and 20.0.....	10	17.1	.28
More than 20.0.....	9	24.2	.26

The average weight, number of nodules, and quantity of nitrogen fixed per plant and the percentage of nitrogen in plants for each experiment and for the entire 217 tests are recorded in table 4. In table 5 is recorded the frequency distribution in nitrogen fixed per plant. The divisions of the data in this latter table are more or less arbitrary.

The mean nitrogen fixed by the 217 cultures was 0.39 mg. and that by the two presumably good strains was 0.47 mg. It is assumed that any culture equaling or exceeding the two control cultures in efficiency should be ranked as "good." It is also arbitrarily assumed that any culture fixing less than 0.30 mg. should be ranked as "poor." Of all cultures examined 55.3 percent were below and 44.7 percent above the mean and are regarded as "inferior" and "superior," respectively; 20.7 percent are classed as "poor" and 27.2 percent as "good."



FIGURE 2.—A, uninoculated alfalfa plants; B, alfalfa plants inoculated with an inefficient strain (192-V) of *Rhizobium meliloti*; C, alfalfa plants inoculated with an efficient strain (A) of *R. meliloti*.

Figure 2 illustrates the difference in growth observed between plants inoculated with "poor" and "good" cultures.

The data presented in table 4 show a surprising variation in the average number of nodules per plant in the different experiments. A careful inspection of the complete data will show, however, that this is accidental in that by chance there were relatively more cultures tested in experiments 6, 8, and 9 that formed a large number of nodules, and hence gave a high average nodule production for those experiments. Three of the four cultures in experiment 9 were high nodule-producing cultures.

Attention has been called by a number of investigators to an apparent negative association between the production of nodules and efficient nitrogen fixation. The data here presented seemed adequate to determine by statistical treatment the presence or absence of such an association. Accordingly, the correlation between these two factors has been calculated for each experiment as well as

for the entire 217 cultures, and the value of  $r$  (correlation coefficient) is recorded in the last column of table 4. Applying a test for significance to these  $r$  values it is found that in the case of experiments 6 and 7 the correlation is significant, i. e., above the 5-percent level, while for experiments 2 and 8, and for the entire 217 cultures, it is highly significant, i. e., above the 1-percent level. On the other hand, the  $r$  values for experiments 3, 4, 5, and 9 are not significant. An analysis of the complete data will reveal a relatively high degree of uniformity in nodule production in experiments 3, 4, and 5, indicating the absence of high nodule-producing cultures; the number of comparisons in experiment 9 is too small to be of any value. In other words, if, in a given series of tests, there happened to be present some cultures of high nodule-producing capacity, a significant negative correlation was found between nodule production and nitrogen fixation. In the absence of (at least a few) high nodule-producing strains no such correlation was found between these two factors.

As further evidence in support of the conclusion reached in the preceding paragraph, the correlation coefficient was calculated between nodules produced and nitrogen fixed by 40 cultures that were retested. These cultures were selected because of wide variation in nitrogen fixation and nodule production and combinations of these two characteristics. The value of  $r$  for these 40 pairs of comparisons was  $-0.523$ , a highly significant value. It seems safe, therefore, to assume that the cultural or strain characteristics of nodule production and nitrogen fixation are definitely negatively associated.

On the other hand, if a comparison is made between the number of nodules produced and nitrogen fixed by a single given strain, a positive correlation is obtained, as is evident from the data presented in table 2 for cultures A and 107.

The available data are inadequate to establish definitely whether low fixation of nitrogen, in the presence of high nodulation, results from or is associated with some physiological alteration in one or both of the symbionts, or whether high nodule-producing and low nitrogen-fixing ability are associated inherent characteristics.

The fact that of 28 individual tests conducted with the 2 control cultures under a wide variety of conditions not one gave nodulation above the level for efficient nitrogen fixation (12.5-15.0 nodules per plant), and that a positive correlation was evident between nodulation and nitrogen fixation in case of the individual strains, might be interpreted as indicating that both inheritance and some physiological factor were operating. In other words, it is possible that a given efficient culture is inherently limited in its ability to produce nodules, but any environmental factor which may tend to raise nodule production into the upper range of a culture's capabilities will result in increased nitrogen fixation. This point is borne out by experience, since abundant nodulation by a "good" strain is considered more valuable than limited nodulation by the same strain.

It is of interest to note that the number of local cultures producing a large number of nodules was not very great. In table 6 the 217 cultures are arranged into 7 groups according to the number of nodules produced. It appears from these data that the number of nodules gives little indication as to nitrogen-fixing efficiency except as the number may be less than or may exceed 12.5 to 15.0 per plant. It has already been suggested that a negative correlation between

nodulation and nitrogen fixation is not evident unless some high nodule-producing strains are present, and the data in table 6 indicate quite clearly why this is true.

There is also some indication in these data that the larger the number of nodules, provided excessive nodulating strains are not involved, the more nitrogen there will be fixed, in that the 10-12.5 nodule group gave the highest average nitrogen fixation.

The data presented in connection with strain 107, table 2, indicate that considerable variation in nodule production is to be expected in some good strains, and the maximum average nodule production recorded for this strain (11.4) is approaching the point of maximum nodulation compatible with efficient fixation. The indications, therefore, are that variations in nodulation up to about 12.5 nodules per plant, as determined in these experiments, is normal for good strains, while the production of nodules in excess of 15 per plant points to an inherent characteristic of high nodule-forming ability associated with low fixation of nitrogen. An examination of the complete data will show that there are sufficient exceptions to this generalization to indicate that other, as yet unidentified, factors may be operating.

#### SUMMARY

An enclosed system of growing alfalfa plants for testing the nodule-producing and nitrogen-fixing efficiency of cultures of rhizobia is described. The method is adapted to both greenhouse and outdoor conditions and requires a minimum of space and attention while plants are growing, and, at the same time, affords a maximum of protection against accidental inoculation. Quantitative measurements of growth, nodulation, and nitrogen fixation are easily attained, and replicated tests as well as repeated tests, at least of "stable" cultures, agree well.

Two hundred and seventeen cultures of rhizobia were isolated from alfalfa and sweetclover in the vicinity of Manhattan, Kans., and their nodule-forming, nitrogen-fixing, and growth-stimulating abilities measured quantitatively by the method described. In comparison with exogenous "good" strains, 27.2 percent of these cultures proved equal or superior, hence, may be classed as "good;" while 20.7 percent were sufficiently inferior to the general average to be classed as "poor."

A comparison of the number of nodules formed with the quantities of nitrogen fixed by the 217 cultures revealed a significant negative correlation between these two characteristics. The evidence indicates, however, that only when the number of nodules formed is relatively high, i. e., more than 12.5 to 15.0 per plant in these tests, does high nodulation become incompatible with efficient nitrogen fixation. Extensive studies with two separate "good" strains gave a positive correlation between nodulation and nitrogen fixation, but in no case did nodulation attain the minimum suggested as being associated with inefficient nitrogen fixation. These facts suggest the inherent association of high nodulation with inefficient nitrogen fixation and low nodulation with efficient nitrogen fixation. Further studies along these lines are highly desirable.





## FACTORS AFFECTING ONION PUNGENCY<sup>1</sup>

By HANS PLATENIUS, *research assistant professor of vegetable crops*, and J. E. KNOTT, *formerly research professor of vegetable crops, New York (Cornell) Agricultural Experiment Station*

### INTRODUCTION

Both mild and pungent onions are demanded for food, the particular use being made of them determining the type required. When onions are used in salads, for seasoning, or as a cooked vegetable, bulbs of relative mildness are usually preferred. On the other hand, a market for highly pungent varieties is created by the manufacturers of such products as sauces, canned soups and extracts, sold in the form of onion powder and onion salt.

The factors influencing the pungency of onions should be of interest to producers and buyers, especially those who use large quantities of this vegetable for manufacturing various food products. Heretofore, no such study has been carried out, mainly because no method was available of measuring small differences in pungency with any degree of accuracy. By means of tasting tests, it is possible to decide whether one particular bulb is stronger than another one, but such a procedure hardly lends itself to an accurate classification of a large number of varieties.

The particular constituent to which the onion (*Allium cepa* L.) owes its pungency was first identified by Lemmler<sup>2</sup> as a volatile oil, known chemically as allyl-propyl-disulfide. Based on these findings, a method was developed by Platenius<sup>3</sup> for determining quantitatively the content of volatile oil in the bulbs. After prolonged hydrolysis, the onion oil is distilled and the total sulfur content is determined in the distillate. The dependability of this method has frequently been checked against tasting tests, and in every instance the differences found noticeable by tasting tests corresponded to differences in the volatile-sulfur content of the bulbs. This fact cannot be considered as conclusive proof of the reliability of the method; still, it is the only confirmation of its value available at the present time.

Preliminary experiments carried out by the writers established the fact that onions differ in pungency not only as a result of genetic factors but also because of soil and other ecological conditions. It was found, for instance, that Italian Red onions imported from Italy were consistently milder than bulbs of the same variety grown in New York. In an earlier publication,<sup>4</sup> the writers also showed that the pungency of onions increases with maturity until the tops begin to fall over. This makes it necessary to compare the pungency of different varieties when the bulbs have reached the same degree of maturity.

<sup>1</sup> Received for publication June 29, 1940. Paper No. 217, Department of Vegetable Crops, Cornell University.

<sup>2</sup> LEMMLER, F. W. DAS ÄTHERISCHE ÖL DER KÜCHENZWIEBEL (*ALLIUM CEPA* L.). Arch. der Pharm. 230: 443-448, 1892.

<sup>3</sup> PLATENIUS, HANS. A METHOD FOR ESTIMATING THE VOLATILE SULPHUR CONTENT AND PUNGENCY OF ONIONS. Jour. Agr. Res. 51: 847-853, illus. 1935.

<sup>4</sup> PLATENIUS, HANS, and KNOTT, J. E. THE PUNGENCY OF THE ONION BULB AS INFLUENCED BY THE STAGE OF DEVELOPMENT OF THE PLANT. Amer. Soc. Hort. Sci. Proc. 33: 481-483, illus. 1936.

The present study was undertaken to classify the most important commercial varieties with respect to pungency and to ascertain to what extent factors such as soil conditions, temperature, moisture supply, and storage influence the pungency of any one variety.

#### RELATIVE PUNGENCY OF DIFFERENT ONION VARIETIES

In the study carried out in 1936 and 1937 a comparison was made of the relative pungency of 16 varieties of onions, including most of the commercially important types. As is commonly known, certain varieties, such as the Bermuda, are poorly adapted to production in northern latitudes because of the unfavorable combination of day length and temperature during the growing season. For that reason, six of the varieties studied were obtained from the Texas substation at Winter Haven,<sup>5</sup> while all the others were grown at Ithaca, N. Y.

At Winter Haven, the usual cultural practices of that region were followed. The soil, a fine sandy loam, received an application of 250 pounds of 11-48-0 fertilizer per acre, and the necessary moisture was supplied by furrow irrigation. The onions were grown from seed, and the seedlings were set out in December. The mature bulbs were harvested in April, shipped to Ithaca by express, and analyzed for volatile-sulfur content within a few days after they were received.

At Ithaca, 10 varieties adapted to northern climates were grown on a gravelly sandy loam, which had received an application of 10 tons of manure and 1,500 pounds of a 5-10-5 fertilizer per acre. The onions were started from seed in February, and the seedlings were transplanted to the field the end of April and harvested during August and September. An overhead irrigation system supplied adequate moisture during periods of insufficient rainfall.

Because it is known that the stage of maturity has an influence on the degree of pungency, care was taken to harvest all varieties when they had developed their maximum content of volatile sulfur, or when the tops began to fall over and wilt. Analyses for volatile sulfur were made within 2 weeks after harvest. A composite sample from at least 25 bulbs was taken for analysis. Dry-weight determinations were made on 100-gm. aliquots dried in a ventilated oven at 60° C. for 48 hours.

The results of this study are presented in table 1, which lists the different varieties in ascending order of pungency based on the determinations made in 1937. Because the data for early varieties grown in Texas are hardly comparable with the data for those from New York, the varieties are grouped separately according to their source.

It will be noticed that, in general, the volatile-sulfur content of the onions was higher in 1936 than in 1937, probably because of differences in the prevailing climatic conditions. At Ithaca the growing conditions were more favorable for the development of the bulbs in 1936 than in 1937, as indicated by a considerable difference in the average weight of the bulbs of each variety at harvest time. More important is the fact that the order, or relative pungency, was the same for all of the early Texas varieties in both years, and that in the late varieties this order was changed only slightly in four of the varieties studied.

<sup>5</sup> The writers are indebted to Mr. L. R. Hawthorn of the Texas Experiment Substation No. 19, Winter Haven, Tex., for supplying the samples in 1936 and 1937.

TABLE 1.—*Volatile-sulfur content on a fresh-weight basis, percentage dry matter, and average fresh weight per bulb of 16 varieties of onions grown at Winter Haven, Tex., and at Ithaca, N. Y., in 1936 and 1937*

Variety and where grown	Results for 1936			Results for 1937		
	Average fresh weight per bulb	Volatile-sulfur content	Dry-matter content	Average fresh weight per bulb	Volatile-sulfur content	Dry-matter content
	<i>Grams</i>	<i>P. p. m.</i>	<i>Percent</i>	<i>Grams</i>	<i>P. p. m.</i>	<i>Percent</i>
Winter Haven, Tex.:						
F. P. I. 106054				165.8	61.1	10.01
Early Grano	210.5	68.1	8.71	206.5	65.2	7.35
Yellow Bermuda	132.6	72.3	10.58	125.9	87.4	8.97
Crystal Wax	134.2	97.9	10.27	106.2	98.4	8.94
White Creole	57.2	120.1	19.20	68.3	127.6	8.63
Red Creole	81.2	155.3	19.40	70.2	143.1	9.69
Ithaca, N. Y.:						
Early Grano	220.1	67.5	6.97	148.7	59.5	7.22
Early Yellow Globe	239.4	94.4	9.53	152.6	72.5	9.82
Utah Sweet Spanish	393.6	97.9	7.89	221.0	78.7	6.87
Red Rocca				205.0	81.1	7.97
Mountain Danvers	306.8	126.6	10.72	143.6	88.2	9.43
Red Wethersfield	268.5	123.0	12.06	146.6	98.3	10.73
Brigham Yellow Globe	265.2	117.3	12.08	176.9	116.2	10.31
Yellow Globe Danvers	244.8	124.5	12.08	151.4	121.5	16.66
Ebenezer	156.0	156.9	14.88	122.0	134.1	13.92
Australian Brown				123.0	144.5	10.13

Early Grano, the only variety grown in both Texas and New York in this experiment, proved to be one of the mildest types in both groups. It was exceeded in mildness only by F. P. I. 106054. Red Rocca, suprisingly, was more pungent than some of the native varieties, although when imported from Italy it is known to be the mildest onion available. This suggests that under certain conditions the influence of soil and climate may be more important than genetic factors in determining the pungency of onions. Early Yellow Globe and the Utah Sweet Spanish are other varieties which, on the basis of these data, must be considered mild. In the medium-pungent groups should be listed the yellow Bermuda, Crystal Wax, Red Wethersfield, Brigham Yellow Globe, and Yellow Globe Danvers. Outstanding as pungent varieties are the Creoles, Ebenezer, and Australian Brown. Obviously these last-named varieties should be given first consideration in selecting stock for manufacturing purposes for which onions of extreme pungency are desired.

From a study of the moisture content of different varieties of onions. Jones and Bisson<sup>6</sup> drew the following conclusion:

Evidently, those varieties that are considered mild and of rather poor storage quality have the highest moisture content, whereas those that are the most pungent and keep best in storage have the lowest moisture content.

This suggests that pungency in any variety is merely a matter of relative concentration of the dry matter, including onion oil. To some extent, this view is supported by the data in table 1, although it must not be overlooked that there are several notable exceptions. Australian Brown and F. P. I. 106054, the strongest and the mildest varieties included in the study carried out in 1937, both had a medium water content of 90 percent.

<sup>6</sup> JONES, H. A., and BISSON, C. S. MOISTURE CONTENT OF DIFFERENT VARIETIES OF ONIONS. Amer. Soc. Hort. Sci. Proc. 31: 165-168. 1934.

In this connection attention should be called to the fact that in these experiments there was fairly good correlation between the average fresh weight of the bulbs and their relative mildness.

## EFFECT OF ENVIRONMENTAL FACTORS ON ONION PUNGENCY

### TEMPERATURE

In 1936 an experiment was conducted in the greenhouse to determine the effect of temperature on the pungency of onions. Thirty plants of Yellow Bermuda were grown in each of several iron drums 22 inches in diameter and 17 inches in depth. The drums were filled with a sandy-loam soil; and by means of an automatic watering device, connected by glass tubing to the base of the drum, a water table 12 inches below the surface was maintained. Two drums were placed in each of three greenhouses, in which the respective temperature ranges were from 50° to 60°, 60° to 70°, and 70° to 80° F. The experiments were started in January and the bulbs were harvested in June. Because it was impossible during the last month of growth to maintain an average temperature below 70° in any of the houses, the data from this experiment must be considered as inconclusive. However, since such striking differences were obtained from the temperature treatments, the results of this experiment are presented in table 2. According to these data, the onions grown at a temperature of from 70° to 80° were three times as pungent as those grown at from 50° to 60°. In evaluating these data, consideration must be given to the physiological age of the plants. At the time the onions were harvested the tops of the plants in the warmest house (70°-80°) had died down completely, whereas those in the coolest house (50° to 60°) were still growing vigorously. Differences in pungency in the three lots can therefore be attributed in part to differences in the stage of maturity. In an earlier paper <sup>7</sup> the writers pointed out that until the time when the tops begin to fall over the pungency of the bulbs increases steadily. Undoubtedly the results of this experiment would have been less striking if the bulbs from the three temperature treatments could have been harvested when the plants had all reached the same physiological age.

An attempt to repeat this experiment in 1937 failed because of serious damage to the plants by red spider and thrips at the high temperature.

TABLE 2.—*Volatile sulfur content on a fresh-weight basis, percentage of dry matter, and average fresh weight per bulb of Yellow Bermuda onions grown in the greenhouse at three different temperatures.*

Growing temperature <sup>1</sup> (°F.)	Average fresh weight per bulb	Volatile-sulfur content	Dry-matter content
	Grams	P. p. m.	Percent
50°-60°.....	65	42.8	8.37
60°-70°.....	74	80.0	7.98
70°-80°.....	46	130.9	7.38

<sup>1</sup> During the last month of growth, the temperature in all houses varied from 70° to 80°.

<sup>7</sup> See footnote 4.

## WATER SUPPLY

Seedlings of Early Grano, Ebenezer, and Utah Sweet Spanish were transplanted to the field late in April 1936. The sandy-loam soil received an application of 10 tons of manure and 1,500 pounds of a 5-10-5 fertilizer per acre. The rows of each variety were so arranged that one-half of each row could be supplied with additional water by overhead irrigation. During the growing season of May 1 to September 1 a total of 9.41 inches of rain fell. One-half of each row received sufficient irrigation to provide in all about 1 inch of water a week, or a total of 17 inches during the growing season of 4 months. When the tops had fallen over, the bulbs were harvested and their content of volatile sulfur determined. The data are presented in table 3. In every instance the bulbs grown with additional water were larger and less pungent than those which had been dependent on the natural rainfall. The reduced pungency of Early Grano and Ebenezer as a result of irrigation was due in part at least to the higher water content of the irrigated bulbs, which tended to reduce the concentration of volatile sulfur in the bulbs when the data were expressed on a fresh-weight basis.

TABLE 3.—*Effect of irrigation on the average weight of bulbs, the content of volatile sulfur on a fresh-weight basis, and the percentage of dry matter of onions of three varieties*

Variety	Water supply	Average fresh weight per bulb	Volatile- sulfur content	Dry- matter content
		<i>Grams</i>	<i>P. p. m.</i>	<i>Percent</i>
Early Grano	(Not irrigated)	154.2	82.5	7.35
	(Irrigated)	220.1	67.5	6.97
Ebenezer	(Not irrigated)	77.5	162.3	15.42
	(Irrigated)	156.0	156.9	14.88
Utah Sweet Spanish	(Not irrigated)	208.4	103.6	7.72
	(Irrigated)	393.6	97.9	7.89

No doubt the effect of irrigation on the pungency of the bulbs depended on the increase in available soil moisture. If the natural rainfall had been higher during that particular season, differences in pungency between irrigated and nonirrigated lots would probably have been negligible.

## SOIL TYPES AND WATER SUPPLY

In the second experiment the influence of soil types and water supply were studied in the greenhouse at an average weekly temperature of 60°–70° F. Iron drums 22 inches in diameter and 17 inches in depth were used as containers for the soil. Four drums were filled with a well-decomposed woody peat, four with a sandy loam, and four with a sandy soil. In two drums of each soil type the water table was maintained 12 inches below the surface; in the remainder it was held at a depth of 16 inches. The water table was controlled by means of an automatic watering device connected by glass tubing to the base of the drum. The low water level provided adequate moisture for the growth of the onions in the peat soil without surface application of water. It was found necessary, however, to add occasionally an equal quantity of water to the surface of each of the drums containing

sandy loam and sandy soil at a low water level in order to maintain growth.

Fertilizer of 5-10-5 analysis was added to each drum at the rate of 1,000 pounds per acre. In October 1936, 30 sets of Yellow Globe Danvers were planted in each drum. Beginning in January 1937 a 15-hour photoperiod was provided by means of Mazda lights. The bulbs were harvested in May 1937, and their content of volatile sulfur was determined. The data in table 4 indicate that the higher content of soil moisture increased the average weight of the bulbs and decreased the volatile-sulfur content when compared with bulbs grown at a low water table. These experiments are in agreement with those obtained outdoors in experiment 1. There was also a marked difference in the pungency of the bulbs, depending on the soil type in which they were grown. The most pungent onions were harvested from the peat, the mildest from the sandy soil.

TABLE 4.—*Effect of soil type and sulfur content and of the water supply on the average weight of bulbs, the content of volatile sulfur on a fresh-weight basis, and the percentage of dry matter of Yellow Globe Danvers onions, grown in the greenhouse*

Soil type	Approximate percentage of sulfur in the soil	Water supply	Average fresh weight per bulb	Volatile-sulfur content	Dry-matter content
	Percent		Grams	P. p. m.	Percent
Peat	0.470	Low	40.4	101.4	12.56
		High	51.8	85.8	12.90
Sandy loam	.039	Low	26.6	80.3	13.19
		High	60.1	74.9	12.84
Sandy soil	.004	Low	41.0	64.0	13.01
		High	50.0	49.5	12.88

The approximate values for the total sulfur content of the three soil types used in this experiment are included in table 4. These data are averages determined for New York soils by Wilson and Staker<sup>8</sup> and by Bizzell.<sup>9</sup> The addition of the equivalent of 1,000 pounds of 5-10-5 fertilizer per acre to the drums did not change these values appreciably.

A striking correlation exists between the pungency of the onions and the soils on which they were grown. However, this relationship is by no means proportional. Whereas the sandy loam contains roughly 10 times and the peat 100 times as much sulfur as the sandy soil, the corresponding volatile-sulfur content of the onions was only 25 and 58 percent higher on the sandy loam and peat than it was on the sandy soil. It should be remembered that the sulfur analyses of the different soil types include both available and unavailable forms of sulfur. These comparisons are based on determinations made for pungency of onions grown with a low water supply. A similar relationship exists between those grown on soils with a high water table.

#### APPLICATION OF SODIUM SULFATE

Drums similar to those used in the previous experiment were filled in duplicate with a well-decomposed peat, a clay loam, and a sandy loam. Bank sand was placed in four additional drums. All were

<sup>8</sup> WILSON, B. D., and STAKER, E. V. THE CHEMICAL COMPOSITION OF THE MUCK SOILS OF NEW YORK. N. Y. (Cornell) Agr. Expt. Sta. Bul. 537, 26 pp. illus. 1932.

<sup>9</sup> BIZZELL, J. A. THE CHEMICAL COMPOSITION OF NEW YORK SOILS. N. Y. (Cornell) Agr. Expt. Sta. Bul. 513, 25 pp., illus. 1930.

fertilized with an equivalent of 1,000 pounds of a 5-10-5 analysis per acre. In addition, two of the drums containing bank sand received an application of sodium sulfate at the rate of 600 pounds per acre. The water table was maintained automatically at a level of 16 inches below the surface in all drums. Only distilled water was used for irrigation. Yellow Bermuda were transplanted, 30 to a drum, in October 1937; the mature bulbs were harvested in May 1938. The temperature of the greenhouse was maintained at 60° to 70° F. and the daily photoperiod lengthened as in the previous experiment. The data in table 5 again show that onions grown on peat soil are not only larger but also considerably more pungent than those from other soil types. The smallest and mildest onions were harvested from sand and sandy loam soil.

TABLE 5.—*Effect of soil type and of sulfate content of the soil on the average weight of bulbs, the content of volatile sulfur on a fresh-weight basis, and the percentage of dry matter of Yellow Bermuda onions grown in the greenhouse*

Soil type	Approximate percentage of sulfur in the soil	Average fresh weight per bulb	Volatile-sulfur content	Dry-matter content
	<i>Percent</i>	<i>Grams</i>	<i>P. p. m.</i>	<i>Percent</i>
Peat	0.470	63.2	157.2	11.11
Loam	.058	48.2	125.2	10.42
Sandy loam	.039	39.3	83.4	10.58
Sand (low sulfate)	.004	20.4	72.8	8.77
Sand (high sulfate)	.008	30.6	77.6	8.70

Again there was a direct relationship between the pungency of the bulbs and the sulfur content of the soils on which they were grown. The addition of an equivalent of 600 pounds of sodium sulfate per acre to the sandy soil doubled the percentage of sulfur present but was insufficient to have an appreciable effect on the pungency of the onions. At any rate, the slight increase in volatile sulfur of the bulbs from sand to which sulfate had been added is probably not significant.

These results lead to the conclusion that on a commercial scale it is not feasible to increase the pungency of onions by the addition of fertilizers of a high sulfur content because the quantities required to produce a noticeable effect is unreasonably large.

#### STORAGE

Experiments to study changes in the pungency of onions held in cold storage were undertaken in 1936 and 1937. Yellow Globe Danvers and Utah Sweet Spanish were stored in bushel crates and kept in an experimental cold-storage room at 33° F. Temperature fluctuations in the room did not exceed  $\pm 1.5^\circ$ . At intervals samples were taken for analysis to determine changes in the content of volatile sulfur and moisture. At the same time, records were taken of the shrinkage that occurred during the different storage periods. Since the bulbs showed neither spouts nor decay none were discarded during the storage period; consequently shrinkage data represent weight losses only.

The results of these experiments are summarized in table 6. It will be noticed that the volatile-sulfur content increased as the storage



period progressed. However, this increase in pungency appeared to proceed at a slower rate toward the end of the storage period; in fact, the lot of Yellow Globe Danvers stored in 1937-38 showed a slight decrease in its content of volatile sulfur.

Shrinkage losses and changes in the percentage of dry matter are partly responsible for variations in the volatile-sulfur content of the different samples. However, table 6 reveals that these two factors alone cannot account for the general tendency of volatile sulfur to increase during the storage period. Instead, it is evident that some of the onion oil was formed during storage from other forms of sulfur and organic constituents in the bulbs.

TABLE 6.—*Shrinkage, content of volatile sulphur on a fresh-weight basis, and percentage of dry matter of onions of two varieties when held in storage at 33° F. for different periods*

Variety	Date sampled	Shrinkage	Volatile-sulfur content	Dry-matter content
		<i>Percent</i>	<i>P. p. m.</i>	<i>Percent</i>
Yellow Globe Danvers	Sept. 17, 1936		110.1	11.52
	Oct. 5, 1936		128.6	11.34
	Nov. 5, 1936		138.2	10.92
	May 15, 1937		130.6	10.09
	Aug. 30, 1937	0.	78.7	6.87
Utah Sweet Spanish	Sept. 28, 1937	1.77	96.3	8.50
	Nov. 4, 1937	2.66	96.8	6.91
	Dec. 21, 1937	7.65	113.0	6.72
	Sept. 27, 1937	0.	114.2	7.11
Yellow Globe Danvers	Nov. 3, 1937	.42	125.2	8.16
	Dec. 20, 1937	2.68	151.4	8.25
	Feb. 22, 1938	3.72	144.2	7.83

## SUMMARY

This study is concerned with the pungency of onions as affected by inherited characteristics, growing temperature, soil type, water supply, and storage. The relative pungency of different samples was measured by determining chemically the volatile-sulfur content of the bulbs and thereby their content of onion oil, which is the substance that gives the onion its characteristic pungent taste and odor.

Of the different factors studied, the inherent characteristics of the varieties examined had the most pronounced influence on pungency. Some varieties were found to contain nearly three times as much volatile sulfur as others. On a dry-weight basis, these differences were much smaller, showing that to some extent relative pungency is merely a question of concentration of dry matter.

An experiment in which onions were grown at different temperatures indicates that the pungency of onions tends to increase as the average temperature is raised. However, the results of this experiment must be considered as inconclusive.

Next to variety, the type of soil on which the onions were grown was the most important factor in determining pungency. Onions grown on a peat soil were roughly twice as pungent as the same variety grown on a sandy soil, while onions on loam or a sandy loam were intermediate in pungency. There was a direct correlation between the volatile-sulfur content of the onions and the total sulfur content of the soil on which they were grown. However, this relationship was

not proportional. Where the percentage of sulfur in sandy loam was about 10 times as high as in sandy soil, the corresponding difference in the volatile-sulfur content of the bulbs was only 25 percent.

Overhead irrigation or a high water table always caused a small, but consistent, lowering of the volatile-sulfur content of the bulbs, and this was associated with a pronounced increase in the size of the bulbs grown with an abundant supply of moisture.

In cold storage, over a period of several months onions slowly increased in pungency. This increase may be accounted for in part by the loss of water and carbohydrates as a result of transpiration and respiration.



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## STERILITY AND ABERRANT CHROMOSOME NUMBERS IN CALORO AND OTHER VARIETIES OF RICE<sup>1</sup>

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### INTRODUCTION

Recent interest in the artificial production of polyploids in various plant species suggested the desirability of a search for similar but naturally occurring chromosomal aberrants in rice. Most of these were sought in the Caloro variety growing at the Biggs Rice Field Station, Biggs, Calif.

In this rice-growing section maximum daily temperatures are high, and the daily range of temperature is often as much as 40° F. during the summer months. Extremes and sudden changes in temperature have been reported to cause chromosomal aberrations in plants, and Matusima (12)<sup>2</sup> reported the finding of tetraploid cells in the root tips of rice seedlings exposed for a short time to temperatures of 42° to 45.5° C.

The stage at which chromosomes are most easily affected by environmental changes is thought to be at the time of sporogenesis. In rice this appears to occur 10 to 15 days prior to heading. In the Caloro variety in California it occurs during and following periods of high temperatures and wide daily ranges in temperature. For example, in 1935, 1936, and 1937, the maximum temperature varied from 100° to 108° F. and the daily range was as high as 47° during the period in which the Caloro variety was forming gametes. It is possible, therefore, that such extremes and ranges in temperature may be responsible in part for the chromosome aberrations reported in this paper. The temperature may act directly upon meiosis, resulting in the production of abnormal gametes similar to those observed by Nandi (29), or it may cause somatic mutations that eventually embrace the germ cells.

Most of the material on which cytological studies were made was collected by the senior writer at the Biggs Rice Field Station, and all cytological studies were made by the junior writer.

The Caloro variety, in which most of the material was collected, is the leading commercial rice in California. It was selected at Biggs in 1913 by E. L. Adams<sup>3</sup> and C. E. Chambliss<sup>3</sup> from Early Watari-bune, a variety of rice (*Oryza sativa* L.) introduced from Japan in the same year by W. K. Brown, of Butte City, Calif. Caloro was distributed and named by the senior writer in 1921. It is a midseason, short-grain variety.

<sup>1</sup> Received for publication December 2, 1940.

<sup>2</sup> Italic numbers in parentheses refer to Literature Cited, p. 396.

<sup>3</sup> Of the Bureau of Plant Industry.

## PREVIOUS INVESTIGATIONS

Prior to 1930, all the cultivated varieties and mutants of rice that had been investigated cytologically had 12 chromosomes in their pollen mother cells. In the last 10 years, however, haploid, aneuploid, triploid, and tetraploid plants also have been discovered and described. In the following tabulation is given a list of the papers published on cytological studies of *Oryza sativa*.

Somatic chromosome number:	References
Diploid 24.....	Kuwada (11) 1910; Nakatomi (27) 1923; Rau (50) 1929; Kato, Kosaka, Hara, Maruyama, and Takiguchi (10) 1930; Selim (52) 1930; Morinaga and Fukushima (16) 1931; Nakamura (25) 1931; Ramiah (42) 1931; Hedayetullah (3) [1934]; Ichijima (9) 1934; Ramiah, Parthasarathi, and Ramanujam (48) 1935; Sakai (55) 1935; Miyazawa (13) 1935; Ramanujam (36) 1935, (37) 1936, (40) 1938; Nandi (28) 1936, (29) 1937; Takahashi (54) 1936; Sethi (53) 1937; Parthasarathy (34) 1938, (35) 1939.
Aneuploids 24+1 or more.....	Nakamori (23) 1932; Ichijima (9) 1934; Ramanujam (38) 1937; Parthasarathy (33) 1938.
Haploid 12.....	Morinaga and Fukushima (17) 1931, (18) 1932, (19) 1934; Ramiah, Parthasarathi, and Ramanujam (45) 1933, (47) 1934; Nakamura (26) 1933; Ichijima (9) 1934; Takahashi (54) 1936.
Triploid 36.....	Nakamori (23) 1932; Ramiah, Parthasarathi, and Ramanujam (46) 1933; Ichijima (9) 1934; Morinaga and Fukushima (20) 1935; Ramanujam (38) 1937.
Tetraploid 48.....	Nakamori (24) 1933; Ichijima (9) 1934; Morinaga and Fukushima (21) 1936, (22) 1937.

## HAPLOID PLANTS

In 1931, Morinaga and Fukushima (17) found a rice plant among  $F_1$  plants of an intervarietal cross between Dekiyama (normal) and Bunkitu-to, a dwarf variety, that proved to be a haploid. In 1933 Nakamura (26), and Ramiah, Parthasarathi, and Ramanujam (45) reported on haploid rice plants, and since then others have been reporting on similar studies (see tabulation above). A small sterile  $F_1$  plant from the cross Blue Rose  $\times$  Carolina Gold was observed at the Biggs Rice Field Station in 1930. This plant was shorter and had narrower leaves, shorter panicles, and much smaller florets than other  $F_1$  plants of the same cross; it was morphologically essentially the same as plants that later proved to be haploids. The collection of verified haploid plants of rice in the United States began in 1934. In that year, H. M. Beachell<sup>4</sup> sent to Washington, D. C., a small sterile plant from a segregating population of a cross between Rexoro  $\times$  Blue Rose, and in 1936 a similar plant from the Latex variety. In 1936, N. E. Jodon<sup>5</sup> also sent to Washington a small sterile plant from C. I.<sup>6</sup> 81C  $\times$  Blue Rose, and the senior writer sent in a similar plant from a pure-line selection (No. 241B7-29) from the Biggs Rice Field Station. These four plants have been

<sup>4</sup> Of the Division of Cereal Crops and Diseases, stationed at Texas Agricultural Substation No. 4, Beaumont, Tex.

<sup>5</sup> Of the Division of Cereal Crops and Diseases, stationed at the Rice Experiment Station, Crowley, La.

<sup>6</sup> C. I. refers to accession number of Division of Cereal Crops and Diseases.

grown in a greenhouse since they were received from the field, and in 1937, 12 univalent chromosomes were found in the prophases of the first reduction division of their pollen mother cells.

These haploid plants grow more slowly and have smaller culms and narrower leaves than normal plants of the varieties or populations from which they arose, and the panicle branches are short and the florets are small and sterile. Haploid No. 241B7-29 often produces a relatively large number of poorly developed parthenocarpic seeds, none of them being viable. The senior writer, with L. L. Davis,<sup>7</sup> found 11 haploidlike plants among the varieties, segregating populations, hybrid selections, and backcrosses growing on an area of less

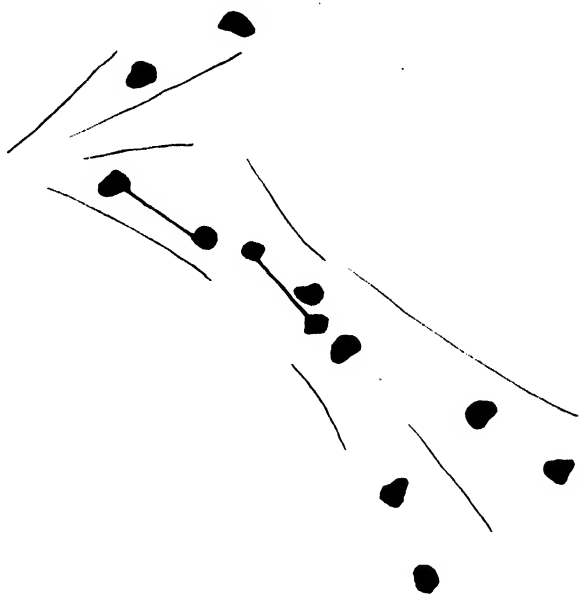


FIGURE 1.—First-division metaphase chromosomes of a pollen mother cell of a haploid mutant (Latex) of *Oryza sativa*.  $\times 3,000$ .

than 1 acre and comprising not less than 300,000 plants at the Biggs Rice Field Station in 1939. Two of these were in the Caloro variety. Since 1934, haploid plants also have been observed from time to time at the rice experiment stations in the Southern States.

The behavior of the chromosomes of haploid plants during meiosis has been described and illustrated by Morinaga and Fukushima (18, 19), Ramiah, Parthasarathi, and Ramanujam (45, 47), and others.

The studies reported here confirm the cytological findings of investigators in Japan and India. It was determined that in the four haploid plants studied the number of bivalent chromosomes at first metaphase never exceeded two (fig. 1); that the synapsis of the bivalents was incomplete; and that split univalents occasionally occurred in first anaphases.

<sup>7</sup> Of the Division of Cereal Crops and Diseases.

## STERILE DIPLOID PLANTS

Single culms of 72 sterile and partly sterile Caloro plants were collected from plots at the Biggs Rice Field Station on October 13, 1938. These plants were fully headed but greener than normal mature plants in the same plots. Sterile and partly sterile rice

plants usually produce additional late tillers early in the fall, apparently utilizing food materials normally required for the developing grain. Such plants can be detected readily in a mature field of rice. Some of the plants collected in 1938 had larger florets than Caloro; in others there was no appreciable difference from normal plants in floret or seed size but some difference in shape. Plants with larger florets than Caloro were highly sterile but produced some parthenocarpic seed. This sort of development was less common, but not absent, in all the partly sterile plants with normal floret size, which



FIGURE 2.—Photomicrograph showing the 12 bivalent chromosomes in pollen mother cells of *Oryza sativa*, variety Caloro.  $\times 800$ .

ranged in fertility from 0.91 to 79.25 percent.

The culms collected were sent to Washington, D. C., and were transplanted in pots in a greenhouse at the Arlington Experiment Farm, Arlington, Va. In the spring of 1939 panicles were collected for cytological studies.

As was expected, most of the plants examined proved to be diploid. Twenty-one of the plants showed a normal chromosome behavior (fig. 2) and the first-division anaphases gave excellent figures for determining their chromosome numbers (fig. 3). The reduction phases of these plants did not differ from those of normal diploid rice varieties described and illustrated by Kuwada (11), Nakatomi (27), Rau (50), Kato et al. (10), and others.

Certain abnormalities in the pollen mother cells and in the behavior of the chromosomes during meioses were observed in 14 diploid plants.

In four of these plants there was a tendency for partial asynapsis of from two to many of the chromosomes, somewhat similar to the condition reported by Ramanujam and Parthasarathy (41). One plant showed very few bivalent chromosomes at first metaphase, a second frequently had as many as 12 univalents at metaphase (fig. 4),

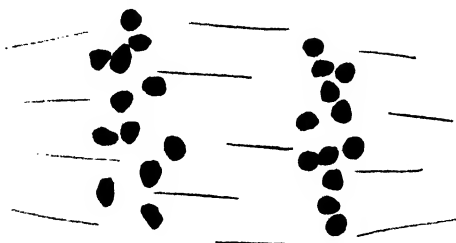


FIGURE 3.—A first-division anaphase from a pollen mother cell of a diploid plant of *Oryza sativa*, variety Caloro.  $\times 3,000$ .

and the remaining two plants generally showed a few unpaired chromosomes in the early phases of the first division. In two of these plants there were no normal anthers. Many of them had been transformed into normal-appearing pistils, and the few anthers present contained very little sporophytic tissue.

Poorly developed sporophytic tissue was characteristic of all the 14 plants that showed abnormal pollen mother cells. There were



FIGURE 4.—A first-division metaphase from a pollen mother cell of a sterile diploid plant of *Oryza sativa*, variety Caloro.  $\times 3,000$ .

few pollen mother cells, and consequently it was hard to find any cells undergoing division. The mother cells in these plants varied greatly in size and in their development failed to show the uniformity characteristic of diploid plants. These conditions limited the studies of chromosomes to the more normal cells. In all 14 plants, the more normal cells showed 24 chromosomes on the first-division anaphase spindle.

Miyazawa (13) described the genetic behavior and gave the chromosome numbers for several types of sterile mutants of rice. Neither in his material nor in these 14 plants was there a change in the chromosome number that might account for the pollen sterility. It is possible, however, that an explanation of the meiotic irregularities observed might be found by a more detailed morphological study of the individual chromosomes.

#### ANEUPLOID PLANTS

Nakamori (23) first reported aneuploid rice plants, and Ichijima (9) reported two heteroploid plants, each with an extra chromosome from progeny of artificially treated material. Parthasarathy (33) also reported on aneuploids, and Ramanujam (38) recently reported on forms having  $2n=24, 25, 26, 27, 28, 29$ , and 30 chromosomes. One panicle was gathered from each of several highly sterile Caloro plants growing in plots at the Biggs Rice Field Station on October 9, 1937. The spikelets of these plants were distinctly larger than those of normal Caloro. The florets of the panicles were sterile, except for an occasional seed and a few poorly developed parthenocarpic seeds that failed to germinate. A few plants, however, were obtained from these seeds, and the pollen mother cells of five of these plants were investigated in 1938.

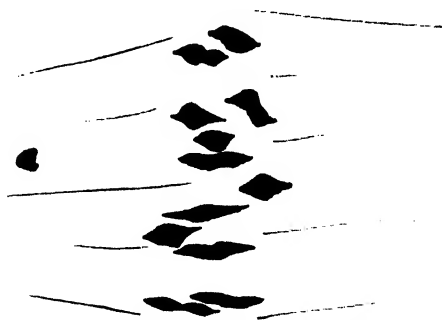


FIGURE 5.—A first-division metaphase from a pollen mother cell of an aneuploid plant ( $2n+1$ ) of *Oryza sativa*, variety Caloro.  $\times 3,000$ .



Of the five plants studied, three had  $2n+2$  and two had  $2n+1$  chromosomes. The presence of one or more extra chromosomes was often indicated in the prophases of the first division when they showed more than the usual 12 chromosome group characteristics of the true diploid. At metaphase the presence of extra chromosomes in the

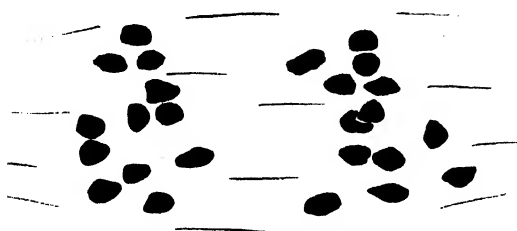


FIGURE 6.—A first-division anaphase from a pollen mother cell of an aneuploid plant ( $2n+2$ ) of *Oryza sativa*, variety Caloro.  $\times 3,000$ .

aneuploid plants was apparent, since at this stage bivalents are readily distinguished from univalents (fig. 5). It was the anaphase, however, that proved to be most useful for making unquestionable counts (fig. 6). Three of these aneuploid plants had 26 chromosomes on the anaphase spindle and two of them 25.

Lack of time and an insufficient number of pollen mother cells from these plants prevented as complete a study of the chromosomes of this group as they deserved.

These aneuploid plants probably originated as crosses between triploid mutants, or partially sterile diploids that produce some gametes with one or more extra chromosomes, and diploid plants growing in the same plots. Ramanujam (38) has reported on trisomics in rice that originated in this manner. Triploid plants are apparently fairly common in the Caloro variety at Biggs, Calif., and it probably would not be very difficult to select from plants obtained from seed of triploids  $\times$  diploids the 12 primary trisomic types. These could then be used in linkage studies.

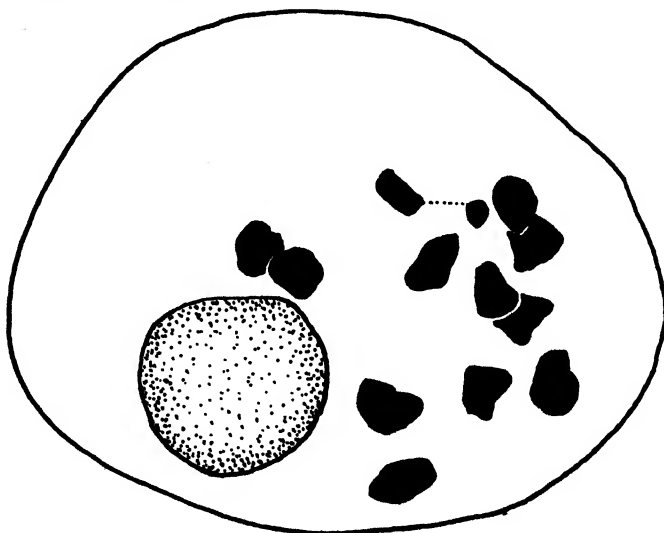


FIGURE 7.—Diakinesis from a pollen mother cell of a triploid plant of *Oryza sativa*, variety Caloro.  $\times 3,000$ .

## TRIPLOID PLANTS

Soon after the discovery of a haploid rice plant, Nakamori (23) and Ramiah, Parthasarathi, and Ramanujam (46) reported on triploid plants. Later, Ichijima (9) found 11 triploids among a group of artificially induced mutations; and Morinaga and Fukushima (20) found triploid plants in considerable numbers in commercial fields.

The chromosomes of the somatic cells, as well as the meiotic phases of triploids, have been illustrated and described by Nakamori (23), Ichijima (9), Morinaga and Fukushima (20), and Ramanujam (38). Meioses have been so well described by Morinaga and Fukushima (20) that there seems to be little more to add from this study except to stress the difficulty of detecting in the early first-division phases the triploid nature of plants.

Nine plants in the Caloro collection of 1938 had the  $2n$  chromosome number 36. In prophases the finding of 12-chromosome groups that showed very little indication of their trivalent nature was the rule (fig. 7).

The trivalent nature of the chromosomes became more apparent at metaphase. At this phase a trivalent often looked like a bivalent with a univalent loosely attached (fig. 8). In some cells at metaphase,

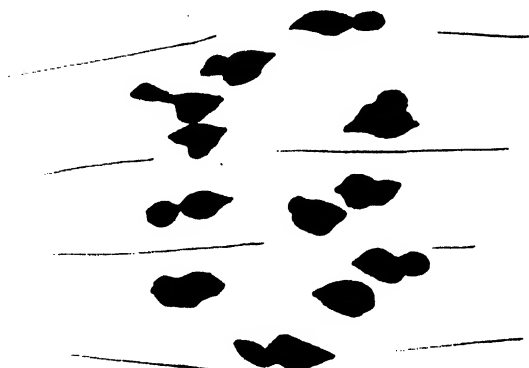


FIGURE 8.—A first-division metaphase from a pollen mother cell of a triploid plant of *Oryza sativa*, variety Caloro.  $\times 3,000$ .

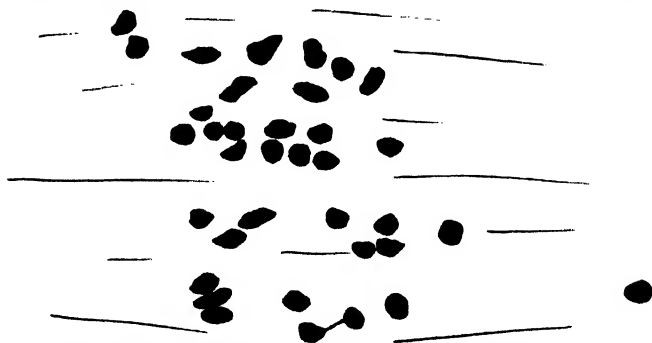


FIGURE 9.—A first-division anaphase from a pollen mother cell of a triploid plant of *Oryza sativa*, variety Caloro.  $\times 3,000$ .

univalents, bivalents, and trivalents were present and distinguishable from one another. However, it was the first-division anaphase that showed all 36 chromosomes distinctly separated on the spindle (fig. 9).

Nine other plants in the same collection were highly sterile in 1938 and had large florets similar to the plants that proved to be triploids, so they, too, apparently were triploids. Both a sterile Early Prolific

plant and a sterile selection in the ninth generations of the cross Colusa  $\times$  Blue Rose had larger florets than the parent varieties at Biggs, Calif., in 1938, and proved to be triploids. In the fall of 1939 several highly sterile plants, similar to the triploids collected in 1938, were again observed in plots of Caloro at the Biggs Rice Field Station.

#### TETRAPLOID PLANTS

Nakamori (24), Ichijima (9), and Morinaga and Fukushima (21) described tetraploid plants of *Oryza sativa* and illustrated both the somatic chromosomes and the chromosomes at meioses.

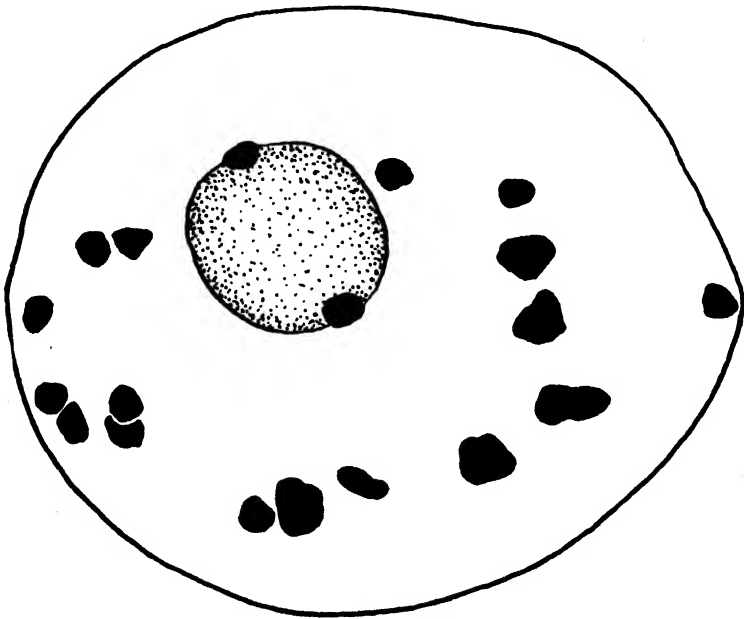


FIGURE 10.—Diakinesis from a pollen mother cell of a tetraploid plant of *Oryza sativa*, variety Caloro.  $\times 3,000$ .

One plant in the 1938 collection of Caloro proved to be a tetraploid. The meiotic behavior in pollen mother cells from this plant was found to be very similar to that described by Morinaga and Fukushima (21). The prophase showed the chromosomes in groups or clumps that differed considerably in size (fig. 10). These groups gave little or no clue, other than their size, to the number of chromosomes they contained. At metaphase, however, the figures had changed; bivalents were most frequent, but in most plates several tetravalent groups were present, looking very much like two bivalents lying side by side (fig. 11).

Trivalents and univalents were exceptional in the early meiotic phases of this tetraploid, and consequently at anaphase the 48 chromosomes usually were equally divided and moved to the poles in a regular manner (fig. 12). Abnormalities in the later phases of meiosis were of infrequent occurrence, and a regular pollen tetrad was characteristic.

In 1939, another tetraploid Caloro plant collected at Biggs, Calif., had larger seeds and longer awns than normal Caloro. The single panicle on this plant, which was growing on infertile soil, produced only 85 seeds and 13 sterile florets; it was therefore 27.1 percent sterile. This plant was similar in all morphological characters to the tetraploid plant reported above, except that the panicle examined had a higher degree of fertility. This probably was due to the fact that a panicle from a tiller was saved from the plant collected in 1938, whereas the panicle of the main culm was collected in 1939.

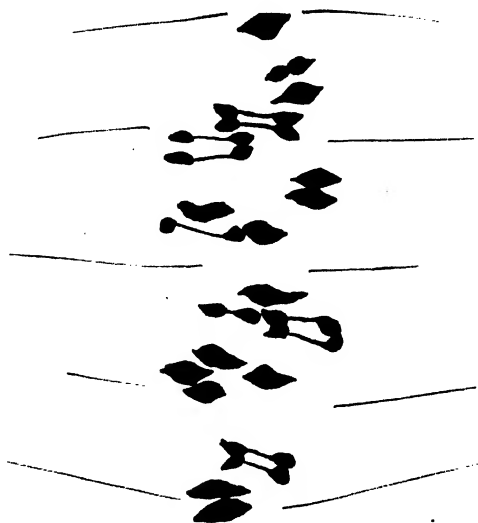


FIGURE 11.—A first-division metaphase from a pollen mother cell of a tetraploid plant of *Oryza sativa*, variety Caloro.  $\times 3,000$ .

#### CELL SIZE IN HAPLOID, DIPLOID, TRIPLOID, AND TETRAPLOID CALORO PLANTS

A study was made of the relative size of the pollen and the guard cells of the stomata in haploid, diploid, triploid, and tetraploid plants, all from the Caloro variety of rice.

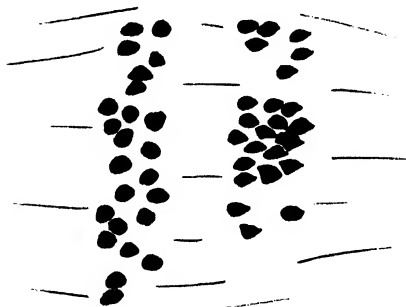


FIGURE 12.—A first-division anaphase from a pollen mother cell of a tetraploid plant of *Oryza sativa*, variety Caloro.  $\times 3,000$ .

Pollen of the four types showed a progressive increase in size from the haploid to the tetraploid, but since there is considerable variation in pollen size in the haploid, triploid, and tetraploid plants, it is believed that the guard cells are more useful for determining the comparative cell sizes. The increase in cell size may be seen in figure 13, A, B, C, and D, which shows the guard cells and a few of the adjoining cells from

haploid, diploid, triploid, and tetraploid plants, respectively, that differ in chromosome numbers by multiples of 12.

In the spring of 1939, diploid, triploid, and tetraploid Caloro plants were subdivided and transplanted, on the same date, in a cage at Arlington Experiment Farm, Arlington, Va. The plants were spaced



FIGURE 13.—Photomicrographs showing the relative size of the guard cells from four plants of *Oryza sativa*, variety Caloro: A, Haploid; B, diploid; C, triploid; D, tetraploid.  $\times 350$ .

about 10 inches apart in rows spaced 1 foot apart and were grown on submerged land under field conditions. Some of the characteristics of these plants are shown in table 1.

TABLE 1.—Characteristics of diploid, triploid, and tetraploid Caloro plants grown at Arlington Experiment Farm, Arlington, Va., 1939

Type	Plants	Average				
		Leaf width		Culms per plant having panicles	Height	Panicle length <sup>1</sup>
		Flag	First leaf below flag			
	Number	Mm.	Mm.	Number	Cm.	Cm.
Diploid	5	13.4	17.2	15.8	111.8	22.8
Triploid	5	14.3	17.5	4.8	110.7	23.6
Tetraploid	8	12.2	15.9	1.6	95.2	17.0

<sup>1</sup> Measured from tip to basal node of panicle.

The meager data presented in table 1 show that the diploid plants were taller and produced many more productive tillers and longer panicles than the tetraploid plants. The triploid plants were nearly

as tall as the diploid; they tillered less but produced somewhat longer panicles. The tetraploid plants had narrower flag leaves than the



FIGURE 14.—Panicles of haploid (A), diploid (B), triploid (C), and tetraploid (D) Caloro plants grown at the Arlington Experiment Farm, Arlington, Va.

diploid or triploid plants. The tetraploid plants were distinctly coarser in all morphological characters and less vigorous and productive than the diploid plants. These polyploid forms appear to be of no practical value (figs. 14 and 15).

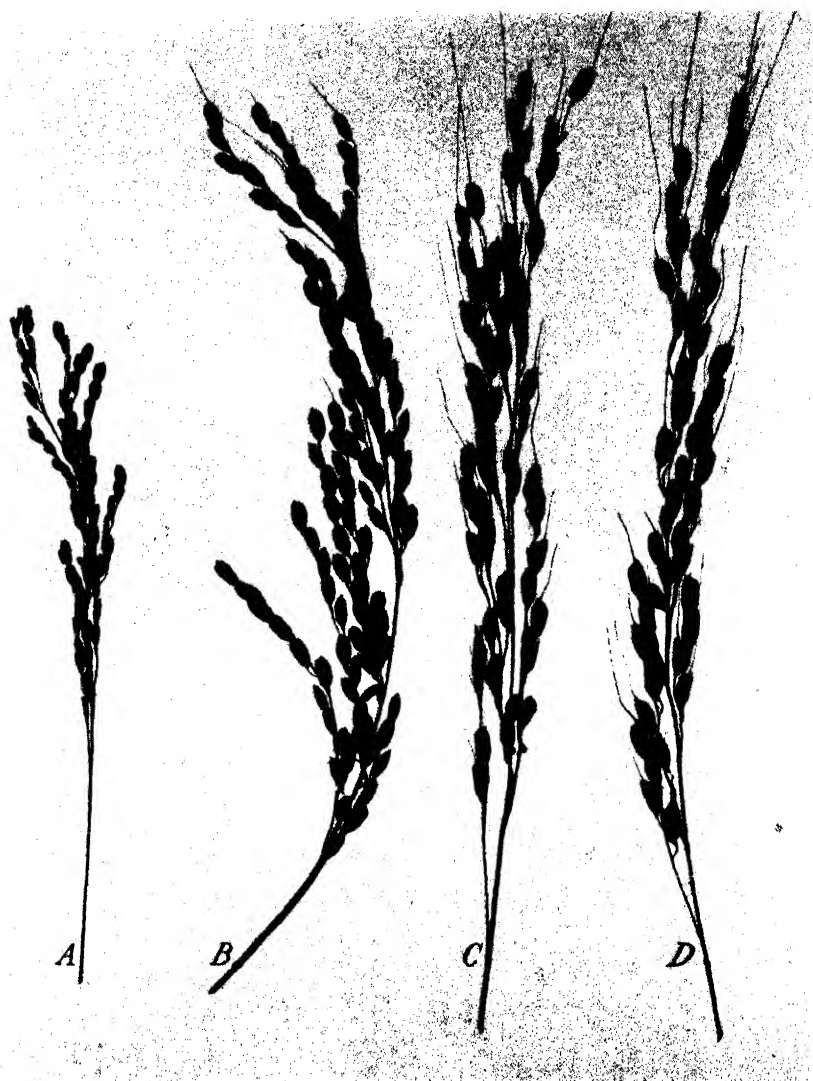


FIGURE 15.—Panicles of haploid (A), diploid (B), triploid (C), and tetraploid (D) Caloro plants grown at the Biggs Rice Field Station, Biggs, Calif., in 1939.

#### CYTOLOGY OF WILD RICE BELONGING TO ORYZA AND OTHER GENERA

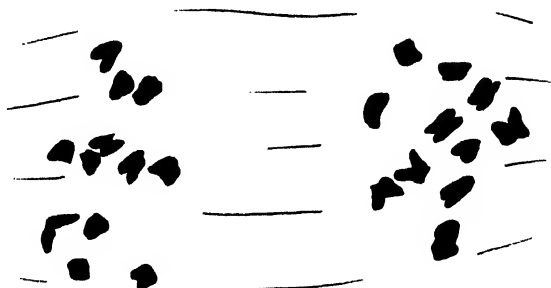
Cytological studies on rice have not been entirely restricted to the cultivated species, (*Oryza sativa*), and a chronological summary of the reported results of chromosome studies in the genus *Oryza* and other genera that have a similar habitat should be of interest and are, therefore, shown in table 2.

TABLE 2.—Chronological list of papers published on cytological studies of wild rice (*Oryza*) and other genera

Species	Somatic chromosomes	Reference
	Number	
<i>Oryza cubensis</i> Ekman	24	Gotoh and Okura (1) 1933.
<i>O. formosana</i> Masamune and Suzuki	24	Do.
<i>O. latifolia</i> Desv. (Cuba)	48	Do.
<i>O. latifolia</i> Desv. (Asia)	24	Ramiah (43) 1934; Heyn (4, 5) 1936, 1938.
<i>O. minuta</i> Presl	48	Morinaga (14) 1934; Ramiah (44) 1936; Nandi (28) 1936; Ramanujam (40) 1938.
<i>O. subulata</i> Nees	24	Horovitz and Pogliaga (8) 1934.
<i>O. longistaminata</i> A. Chev. and Roehr	24	Ramiah et al. (49) 1935; Ramanujam (40) 1938.
Do.	48	Ramiah et al. (49) 1935.
<i>O. officinalis</i> Wall.	24	Nandi (28) 1936.
<i>O. barthii</i> A. Chev.	24	Heyn (4, 5) 1936, 1938; Ramanujam (40) 1938.
<i>O. meyeriana</i> Baill.	24	Heyn (4, 5) 1936, 1938.
<i>O. sativa</i> var. <i>spontanea</i>	24	Do.
<i>O. glaberrima</i> Steud.	24	Ramanujam (40) 1938.
<i>O. coarctata</i> Roxb.	48	Parthasarathy (34) 1938.
<i>Zizania aquatica</i> L.	30	Nandi (28) 1936; Longley <sup>1</sup> 1938; Ramanujam (40) 1938.
<i>Z. latifolia</i> Turch.	34	Hirayoshi (7) 1937.
Do.	30	Ramanujam (40) 1938.
<i>Leersia japonica</i> Hack.	60	Hirayoshi (7) 1937.
<i>L. japonica</i> Makino	96	Do.
<i>L. oryzoides</i> Sw.	48	Ramanujam (40) 1938.
<i>L. herandra</i> Sw.	48	Hirayoshi (7) 1937; Ramanujam (40) 1938.
<i>Hydrolyza aristata</i> Nees	24	Do.
<i>Chikusichloa aquatica</i> Koidz	24	Hirayoshi (7) 1937.
<i>Ligeum spartum</i> L.	40	Ramanujam (40) 1938.

<sup>1</sup> Unpublished data.

The only chromosome determinations made in this study, other than on forms of *Oryza sativa*, were from the pollen mother cells of *O. latifolia* Desv. from Cuba and of the same species from the Botanical Gardens of the Straits Settlements, British Malaya. The specimen

FIGURE 16.—A first-division anaphase from a pollen mother cell of a diploid form of *Oryza latifolia*.  $\times 3,000$ .

studied of *O. latifolia* from Cuba has the tetraploid chromosome number 48, as reported also by Gotoh and Okura (1). *O. latifolia* from the Straits Settlements has the diploid chromosome number 24 (fig. 16), as previously reported by Ramiah (44) and Heyn (4, 5).

The chromosomes of the tetraploid plant of *Oryza latifolia* were all bivalents at the first-division prophase (fig. 17), and the later meiotic



phases showed a regular distribution of 24 chromosomes to each of the four cells of the pollen tetrad.

Plants of the tetraploid form of *Oryza latifolia* (from Cuba) are much more vigorous and have larger culms, leaves, panicles, and somewhat larger seeds than the diploid form from Asia.

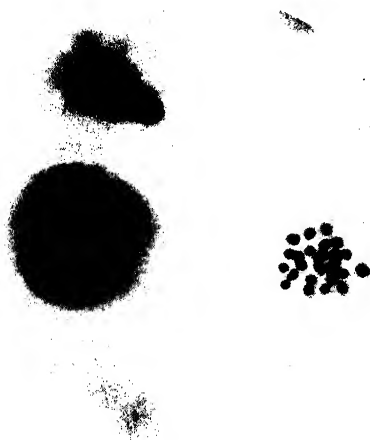


FIGURE 17. Photomicrograph showing metaphases in the pollen mother cells of a tetraploid form of *Oryza latifolia*.  $\times 500$ .

#### INTERSPECIFIC HYBRIDS IN RICE

In 1931, Ramiah (42) reported attempts to cross *Oryza sativa* with the wild (diploid 24) species *O. longistaminata* A. Chev. and Roehr. and *O. latifolia* (from Asia), but only parthenocarpic seed were obtained. Later, however, Ramiah (44) reported that a successful cross had been made between *O. sativa* and *O. latifolia* (from Asia). A chronological list of the interspecific crosses reported with *Oryza* is shown

in table 3. Gotoh and Okura (2) reported on crosses of *O. sativa*  $\times$  *O. cubensis* Ekman, and *O. sativa*  $\times$  *O. latifolia* (from Cuba). The senior writer also made the cross Colusa (*O. sativa*)  $\times$  *O. cubensis* in 1933; the  $F_1$  plants were sterile and more like *cubensis* than Colusa. This agrees with the observations of Gotoh and Okura (2). The  $F_1$  plants were backcrossed to Blue Rose (*O. sativa*), and from this progeny and progeny from natural crosses with *O. sativa* some lines rather resistant to *Helminthosporium oryzae* Van Breda de Haan have been isolated. Interspecific crosses in rice appear to be easier to make when *O. sativa* is used as the female parent. Morinaga and Aoki (15) have also shown that certain cultivated varieties cross more readily with wild rice than do others.

TABLE 3.—Chronological list of reported interspecific crosses in *Oryza*

Cross	Somatic chromosomes	Reference
	Number	
<i>O. sativa</i> $\times$ <i>latifolia</i> (Asia) .....	24 $\times$ 24	Ramiah (43) 1934.
<i>O. sativa</i> $\times$ <i>latifolia</i> (Cuba) .....	24 $\times$ 48	Gotoh and Okura (2) 1935; Hirayoshi (6) 1937; Morinaga and Aoki (15) 1938.
<i>O. latifolia</i> (Cuba) $\times$ <i>sativa</i> .....	48 $\times$ 24	Morinaga and Aoki (15) 1938.
<i>O. sativa</i> $\times$ <i>cubensis</i> .....	24 $\times$ 24	Gotoh and Okura (2) 1935.
<i>O. sativa</i> $\times$ <i>minuta</i> .....	24 $\times$ 48	Morinaga (14) 1934; Okura (32) 1937; Morinaga and Aoki (15) 1938.
<i>O. minuta</i> $\times$ <i>sativa</i> .....	48 $\times$ 24	Nandi (31) 1938; Morinaga and Aoki (15) 1938.
<i>O. sativa</i> $\times$ <i>officinalis</i> .....	24 $\times$ 24	Nandi (31) 1938, Ramenujam (39) 1937.
<i>O. minuta</i> $\times$ <i>officinalis</i> .....	48 $\times$ 24	Nandi (31) 1938-36.
<i>O. minuta</i> $\times$ <i>latifolia</i> (Cuba) .....	48 $\times$ 48	Morinaga and Aoki (15) 1938.

In the Annual Report of the Paddy Breeding Station, Coimbatore, India, for the year 1928-29, page 400, a statement indicates that a cross between *Oryza sativa*  $\times$  *O. longistaminata* was obtained, and the nature of the  $F_1$  is indicated. In a similar report for the year 1933-34, mention is made of  $F_2$  plants of such a cross.

In all interspecific crosses reported to date with *Oryza sativa*, the  $F_1$  plants were either completely sterile or were very low in fertility. The  $F_1$  plants of the cross *O. latifolia*  $\times$  *O. minuta* and reciprocal, both tetraploid species, according to Morinaga and Aoki (15), were also relatively low in fertility. Ramanujam (39) planted self-sterile  $F_1$  plants of the cross *O. sativa*  $\times$  *O. officinalis* (diploids) close to *O. sativa* plants, and the plants set 10 seeds, probably by cross-pollination of the  $F_1$  with *O. sativa*. Six of the 10 seeds germinated and produced sterile plants, all of which proved to be triploids with the  $2n$  chromosome number 36. This was assumed to be due to the formation of diploid gametes by double division of the univalent chromosomes in the  $F_1$  plants.

#### DISCUSSION

The fact that sterility often occurs in intervarietal crosses in cultivated rice, even though the parents have the same number of chromosomes, and in the  $F_1$  plants of all the interspecific crosses with *Oryza* thus far reported, indicates that the chromosomes of the cultivated varieties have probably undergone changes due to gene mutations and genic rearrangement to such an extent that they often are incompatible when crossed. A knowledge of some of the chromosomal aberrations, gene mutations, and apparent genic rearrangements, mentioned in this paper and in the literature cited, that have occurred in cultivated varieties in a relatively short period of time tends to confirm this point of view. It is possible that the cultivated rice varieties classed by Kato et al (10) as *O. sativa japonica* and *O. sativa indica* represent varieties of the same origin that have been grown under different environmental conditions so long that, owing to an accumulation of gene mutations and genic rearrangements within the chromosomes, they now are largely incompatible when crossed.

Haploid rice plants probably arise from the parthenogenetic development of an egg cell, as appears to be the case with most haploids in higher plants. Aneuploids have been shown to arise in rice by pollination of self-sterile triploid by diploid plants (23). The aneuploids reported in this paper also are believed to have originated in this way, because the maternal plants were very similar to plants that later proved to be triploids. Triploids are believed to originate by the union of haploid and diploid gametes; by the fusion of a diploid egg and a haploid male gamete, as in a cross of tetraploid  $\times$  diploid plants; or by fertilization of a haploid egg by two male nuclei (dispermy). Diploid gametes have been reported in *Oryza sativa* by Nandi (29, 30), and by Ramanujam (39); and also in *O. officinalis* by Nandi (31). Tetraploid plants may arise by the fusion of diploid gametes or by somatic chromosome doubling. Ramanujam (39), in a cross of *O. sativa*  $\times$  *officinalis*, obtained a sterile  $F_1$  hybrid; from the  $F_1$  backcrossed to *O. sativa*, six triploid plants were obtained. Cytological studies of the triploids indicated the presence of two sets of *O. sativa* chromosomes and one set of *O. officinalis*, which according to Ramanu-

jam (39, p. 252) "is the first case in plants of experimental demonstration of the origin of polyploidy through formation of diploid gametes by double division of the chromosomes."

This chromosome study of the rice varieties grown in the United States has shown that there are present in them nearly all the aberrant forms that have been described in rice in India and Japan. However, it offers neither confirmation nor denial of the view put forth by Kuwada (11) and Sakai (51) and supported by Nandi (28) that the basic chromosome number for rice is 5. Ramiah, Parthasarathi, and Ramanujam (47) found some pollen mother cells in a haploid plant with six bivalent chromosomes at diakinesis, a discovery that does not exclude the possibility that 6 may be the basic number. The X-ray studies of Ramiah, Parthasarathi, and Ramanujam (48) have shown that rings and chains of four chromosomes result from induced transmutations. If such groupings are readily produced by induced changes, why, as Morinaga and Fukushima (19) ask, do the chromosomes of the haploids fail to show more bivalents and larger chromosome groups in the meiotic divisions if the basic number for rice is 5?

The early prophase threads of rice chromosomes do not seem very promising for a morphological study of the individual chromosomes. It is unfortunate, however, that lack of time and the difficulty of obtaining good material of sterile diploids and of trisomics prevented a more careful search in these groups for such morphological features as might explain the aberrant behavior of the pollen mother cells in pollen-sterile plants. A careful examination of chromosomes might disclose characteristics that would be useful in determining which chromosomes were present in triplicate in the trisomic plants.

#### SUMMARY

Haploids in rice found at several experiment stations, and haploid, sterile diploid, aneuploid, triploid, and tetraploid plants in the Caloro variety of rice that arose as mutations at the Biggs Rice Field Station, Biggs, Calif., are described briefly. The polyploid types appear to be of no practical value.

Cytological studies of these mutants show the presence in the United States of nearly all the aberrant forms reported in rice in Japan and India.

Comparative studies of the gametic tissue of normal and sterile diploid plants have shown that the sterile plants have an abnormal meiotic behavior despite their diploid chromosome number.

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# KNOB POSITIONS ON TEOSINTE CHROMOSOMES<sup>1</sup>

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## INTRODUCTION

The meiotic prophases of corn (*Zea mays* L.) and its near relatives are unusually favorable for critical studies of the morphological characters of the chromosomes, since the chromosomes at the early thread stages of the pollen mother cells are sharply differentiated by iron-aceto-carmin stain. It is on these paired threads, before contraction sets in to obscure the finer markings of the pachytene chromosomes, that knobs are most prominent, and it is the presence of these and other distinctive characters visible on the earlypro phase chromosomes that has made this group of plants so useful for chromosome studies.

The writer (7)<sup>2</sup> has recently published on the relationship between knob position and knob frequency in corn chromosomes, showing that each chromosome arm has a most favorable knob-forming position and that the frequency of occurrence of a knob depends upon its proximity to this most favorable position. The available data suggested a similar relationship for the knobs on annual teosinte (*Euchlaena mexicana* Schrad.) chromosomes. Recently additional data on the morphological characters of teosinte chromosomes have been obtained and have been analyzed with respect to the frequency with which knobs are found at different loci on the chromosomes. These data substantiate the earlier suggested relationship between knob position and knob frequency.

These data also seem to show a division of all teosintes from Guatemala into two groups, a northern group centering around Huehuetenango, and a southern group centering around Progreso and Jutiapa.

Finally, these data indicate that the chromosomes of the northern group of teosinte are more like corn than those of the southern group.

## MATERIAL AND METHODS

The material for this study of the morphological characters of the midprophase chromosomes of Guatemalan teosinte was taken from plants grown in the greenhouse at Washington, D. C., at Charleston, S. C., at Chapman Field and Canal Point, Fla., and at Torrey Pines, Calif.

The seeds for these plantings came from Nojoyá and San Antonio Huixta, in the Department of Huehuetenango, northern Guatemala; and from Moyuta, Progreso, Jutiapa, Retana, and Chilamates, in the Department of Jutiapa, southern Guatemala (4, map). The seeds from the Department of Huehuetenango will be referred to as the northern group, and the seeds from the Department of Jutiapa as the southern group. In the latter group is also included Florida teosinte, which

<sup>1</sup> Received for publication December 2, 1940.

<sup>2</sup> Italic numbers in parentheses refer to Literature Cited, p. 413.



historical evidence indicates originated near Progreso, in southern Guatemala.

The methods of preparing and studying pollen mother cells were the same as those outlined recently (7).

### CHROMOSOME LENGTH

The length of any chromosome depends upon the stage at which it is measured. During the prophase of the first reduction division the chromosomes of teosinte change from the long threads of the early prophase to the compact bodies found at diakinesis. This change frequently reduces the length of a chromosome from  $80\mu$ – $100\mu$  to  $8\mu$ – $10\mu$ . Care was taken to eliminate as much variation in chromosome length as possible, but in spite of all precautions the measurements varied much. The means for each chromosome (table 1), although derived from many measurements, still show rather large errors.

It is only after months of study that each of the 10 corn chromosomes can be identified in the early thread stage. This problem is even more difficult with teosinte chromosomes, since knobs, the positions of which help materially in identifying corn chromosomes, are all terminal in teosinte. Identification, therefore, of the teosinte chromosomes depends upon their relative lengths, the relative length of the two arms of each, and upon other minor but stable characteristics that continued study has shown to distinguish certain chromosomes.

The homology between teosinte and corn chromosomes has been observed in a previous study (5) of  $F_1$  teosinte-corn hybrids, in which it was found that the numbers assigned to corn chromosomes, based on total length, are with few exceptions equally applicable to teosinte chromosomes.

The extended threadlike chromosomes of both teosinte and corn are found to differ in length, in addition to the usual variation, because of the presence of knobs. The increase in length of a corn chromosome due to the presence of a knob has been previously suggested (5), but it has been tested only recently by actual measurements. From this test it was found that each of the 15 chromosome arms known to bear knobs is appreciably shorter when knobs are absent than when knobs are present (table 1). The writer suspects that some of this increase in length of knob-bearing arms may be mechanical. The elastic of a slingshot stretches when a stone is in it and in somewhat the same manner the longer chromosome arms may be stretched by the presence of a knob. Such a mechanical increase in chromosome length may account for the fact that in  $F_1$  teosinte-corn hybrids (5) the terminal knobs of the teosinte homologue were frequently seen reaching beyond the end of the corn chromosome.

At the time the measurements given in table 1 were made it was not considered that differences in chromosome length might be due at least in part to the presence or absence of a knob. Since the teosinte material collected had relatively few arms without knobs, an estimate of what the length of the arms would be without knobs was obtained by subtracting from the mean length the mean knob size for the particular arm.

TABLE 1.—Mean length and position of fiber attachment of midprophase chromosomes

Chromosome No.	Measurements for —		Mean length for —		Mean length (without knobs) for —		Mean fiber-attachment position for —		Mean fiber-attachment position (without knobs) for —	
	South- ern teo- sinte	North- ern teo- sinte	Corn	Northern teosinte	Corn	South- ern teo- sinte	North- ern teo- sinte	Corn	South- ern teo- sinte	North- ern teo- sinte
	Number	Number	Number	Number	Number	Number	Number	Number	Number	Number
I.	90	126	343	80.97±1.61	82.40±0.90	78.60	79.58	79.74	0.422	0.425
II.	68	133	306	70.50±1.46	66.50±1.25	66.82	65.21	63.43	0.437	0.427
III.	53	120	354	62.36±1.20	62.00±1.74	59.79	61.02	60.01	0.330	0.326
IV.	55	115	342	61.11±1.64	60.31±1.29	57.97	57.64	57.03	0.331	0.326
V.	49	111	320	58.58±1.85	58.02±1.16	54.47	55.41	58.57	0.382	0.379
VI.	80	133	377	43.65±1.91	45.64±1.79	41.38	44.07	46.61	0.473	0.469
VII.	105	129	367	47.98±1.87	48.75±1.51	45.46	46.03	44.42	0.229	0.241
VIII.	112	114	361	47.84±1.93	47.48±1.51	45.16	43.86	44.79	0.270	0.269
IX.	113	135	416	42.55±1.77	42.88±1.75	40.87	40.68	41.51	0.309	0.274
X.	112	169	391	37.50±1.70	37.33±1.50	35.03	37.06	36.93	0.264	0.267
									0.352	0.333
									0.263	0.267

1 Length of short arm relative to entire chromosome including knobs.

2 Length of short arm relative to entire chromosome excluding knobs.

All recent studies of knobs have included, when possible, an indication of the size of the individual knobs, according to 10 steps for classification, beginning with the smallest, having a diameter of  $0.33\mu$ , and ending with the largest, with a diameter of  $3.3\mu$ . From this classification the mean size of each knob and the mean size per plant of each knob were calculated and are given in table 2, columns 9 and 10, and 11 and 12, respectively. The mean size per plant is the number of microns that each knob increases the length of the arm on which it occurs. Thus, the length of each chromosome, excluding the knobs, was computed from the total length (table 1). Such a calculation disregards the mechanical elongation of a chromosome due to the presence of knobs, if such elongation has occurred.

TABLE 2.—Mean size and frequency of chromosome knobs in Guatemalan teosinte

Chromosome	No.	Arm	Measurements for—				Knob frequency in—		Mean knob size for—		Mean knob size per plant at each position for—	
			Southern teosinte		Northern teosinte		Southern teosinte	Northern teosinte	Southern teosinte	Northern teosinte	Southern teosinte	Northern teosinte
			With knob	Without knob	With knob	Without knob						
			Number	Number	Number	Number	Percent	Percent	$\mu^1$	$\mu^1$	$\mu^1$	$\mu^1$
X		Short	0	53	0	68	0	0	0	0	0	0
VIII		do	0	47	55	1	0	98.21	0	1.97	0	1.93
VI		do	0	45	53	8	0	86.89	0	2.09	0	1.82
VII		do	0	53	61	1	0	98.39	0	2.17	0	2.14
IX		do	41	0	55	0	100.00	51.47	2.57	2.03	2.57	2.03
III		do	27	0	60	0	100.00	100.00	1.89	1.49	1.89	1.49
IV		do	53	0	35	33	100.00	51.47	2.47	.53	2.47	.27
V		Long	32	0	50	0	100.00	100.00	2.16	1.45	2.16	1.45
IX		Long	52	1	8	54	98.11	12.90	2.73	.48	2.68	.06
II		Short	33	0	62	0	100.00	100.00	2.14	1.85	2.14	1.85
V		Long	31	1	46	4	96.87	92.00	2.01	1.26	1.95	1.16
VII		do	45	0	18	43	100.00	29.51	2.52	1.15	2.52	.34
I		Short	43	0	50	0	100.00	100.00	1.92	1.37	1.92	1.37
VIII		Long	36	11	44	12	76.60	78.57	.89	1.03	.68	.81
IV		do	20	7	56	4	74.07	93.33	1.69	1.27	1.25	1.18
VI		do	38	0	64	0	100.00	100.00	2.07	1.57	2.07	1.57
II		do	32	1	59	3	96.97	95.16	1.66	1.57	1.61	1.49
III		do	0	41	7	49	0	12.50	0	.66	0	.08
I		do	0	43	1	58	0	1.69	0	.33	0	.01
Mean per knob									2.05	1.35	1.99	1.17
Mean per plant											25.91	21.05

<sup>1</sup> Approximate diameter of knob.

These efforts to find the length of each chromosome, exclusive of knobs, are not intended to suggest that knobless chromosomes represent the characteristic chromosomes of teosinte or corn. They were made in order to arrive at a basic length that would prove most useful in comparing chromosomes from the two teosinte groups with each other and with corn.

The mean chromosome lengths, both with and without knobs, are given in table 1 for all chromosomes of the two teosinte groups and of corn. These data show that the chromosome with the greatest length difference is VI. It had been found in an earlier study (5) of  $F_1$  teosinte-corn hybrids that chromosomes VI from teosinte and corn

are equal in length or that chromosomes VI of teosinte may be even longer than chromosome VI of corn, but never shorter as the lengths in table 1 would indicate. Consequently, it would seem that the data presented in table 1 on chromosome lengths give no definite proof that there is a significant difference in the lengths of any of the 10 homologues as they appear in either of these two teosinte groups and in corn.

The relative length of the short arm of each chromosome to its whole length is given in table 1 under the heading "Mean fiber-attachment position." When measurements that included the length of the knobs were used in determining these fractions for the two teosinte groups and for corn, it was found that in some cases the three homologous chromosomes had quite different positions for the fiber attachment.

However, when these fractions were determined from measurements from which the length of the knobs had been deducted, the large differences in fiber-attachment positions for each homologue were reduced, and the whole array of fiber-attachment positions given in the last three columns of table 1 suggests, with the possible exceptions of chromosomes IX and VIII, no real differences between the chromosomes from either of the teosinte groups or from corn.

If the mean lengths of each chromosome arm of the two teosinte groups are compared (table 3), it will be seen that the mean lengths from measurements without knobs show only those for the long arm of chromosome VIII to be appreciably different. When this comparison includes corn as well, there are found to be differences in the long arms of chromosomes VIII, VI, V, and II and the short arm of V.

TABLE 3.—Mean length of arms of midprophase chromosomes of teosinte and corn

Chromosome		Length (with knobs) for—			Length (without knobs) for—		
No.	Arm	Southern teosinte	Northern teosinte	Corn	Southern teosinte	Northern teosinte	Corn
X.	Short.	$9.22 \pm 0.21$	$9.91 \pm 0.18$	$9.81 \pm 0.10$	$9.22$	$9.91$	$9.81$
VIII.	do.	$11.79 \pm .28$	$14.38 \pm .30$	$11.26 \pm .15$	$11.79$	$12.45$	$11.26$
VI.	do.	$10.01 \pm .26$	$11.00 \pm .25$	$11.91 \pm .15$	$10.01$	$11.00$	$11.91$
VII.	do.	$12.94 \pm .25$	$14.60 \pm .26$	$12.44 \pm .16$	$12.94$	$12.78$	$12.42$
IX.	do.	$12.53 \pm .23$	$14.21 \pm .30$	$15.21 \pm .18$	$12.53$	$12.07$	$13.82$
III.	do.	$22.32 \pm .47$	$21.03 \pm .46$	$20.51 \pm .26$	$19.75$	$19.90$	$20.35$
IV.	do.	$23.88 \pm .74$	$23.04 \pm .53$	$22.47 \pm .15$	$21.99$	$21.55$	$22.43$
X.	Long	$28.28 \pm .54$	$27.42 \pm .37$	$27.12 \pm .28$	$25.81$	$27.15$	$27.12$
V.	Short	$27.08 \pm .94$	$26.78 \pm .59$	$27.37 \pm .35$	$25.52$	$25.33$	$27.37$
IX.	Long	$30.02 \pm .59$	$28.67 \pm .52$	$28.03 \pm .30$	$27.34$	$28.61$	$27.69$
II.	Short	$31.36 \pm .72$	$29.71 \pm .59$	$29.51 \pm .35$	$29.22$	$27.86$	$28.60$
V.	Long	$30.90 \pm .95$	$31.24 \pm .63$	$32.45 \pm .38$	$28.95$	$30.08$	$31.20$
VII.	do.	$35.04 \pm .68$	$34.19 \pm .60$	$34.34 \pm .39$	$32.52$	$33.85$	$32.00$
I.	Short	$35.05 \pm .75$	$35.18 \pm .74$	$35.87 \pm .39$	$33.13$	$33.81$	$34.88$
VIII.	Long	$36.05 \pm .71$	$32.22 \pm .60$	$36.22 \pm .42$	$35.37$	$31.41$	$33.53$
IV.	do.	$37.23 \pm 1.03$	$37.27 \pm .83$	$36.31 \pm .41$	$35.98$	$36.09$	$34.60$
VI.	do.	$33.64 \pm .78$	$34.64 \pm .63$	$36.82 \pm .39$	$31.57$	$33.07$	$34.70$
II.	do.	$39.21 \pm .83$	$38.84 \pm .72$	$38.97 \pm .45$	$37.60$	$37.35$	$34.83$
III.	do.	$40.04 \pm .79$	$41.20 \pm .82$	$41.49 \pm .52$	$40.04$	$41.12$	$39.66$
I.	do.	$45.47 \pm .95$	$45.78 \pm 1.26$	$46.52 \pm .55$	$45.47$	$45.77$	$44.86$

A summary of the data on length suggests a possible difference in lengths of chromosomes VIII, VI, V, and II between both groups of Guatemalan teosinte and corn. However, when the teosinte and corn chromosomes were within the same cell of an  $F_1$  teosinte-corn hybrid, there was no difference in length between chromosome VI of corn and its homologue in teosinte.

## CHROMOSOME KNOBS

It was the determination of knob frequency and position that revealed morphological differences in the chromosomes of corn from different geographical regions. Measurements have failed to show real differences in length between homologous chromosomes of teosinte from northern and from southern Guatemala. Consequently, a study has been made of the number, frequency, size, and position of the knobs on the chromosomes of these two teosinte groups.

All knobs on Guatemalan teosinte chromosomes are terminal. The few suggestions of internal knobs are never more prominent than large chromomeres and consequently are disregarded in this discussion.

Since the short arm of chromosome VI has no true knob in either teosinte or corn, but only a dark-staining body that is always present and is associated with the nucleolus organizer, this arm has been omitted in much of this consideration of chromosome knobs. Thus there are left 19 arms for examination. In northern Guatemalan teosinte, 18 of these arms have been seen with a terminal knob, while in teosinte from southern Guatemala only 13 have been found terminated by a knob.

The frequency of occurrence of each of the 13 knobs on the chromosomes of the southern group was found to be relatively high, and only 2 knobs have frequencies below 90 percent. In the northern group the frequency is more variable, although 13 of the 18 knobs have frequencies above 75 percent, the remaining 5 knobs occurring much less frequently. Table 2 shows the frequency of occurrence of each knob in each of the teosinte groups.

A comparative study of the first few plants from the two teosinte groups suggested that the knobs on the chromosomes of the southern group were much more prominent than those on the chromosomes of the northern group. To verify this suggested difference in knob size, the measurements of table 2 were made. These measurements are somewhat indefinite, since knobs are not spherical, nor even constant in shape; consequently it was the amount of knob material present and not in all cases the actual diameter or length of the longest axis that placed a knob in its size class.

According to this classification, the mean knob size for the northern group is  $1.35 \mu$ , while that of the southern group is  $2.05 \mu$  in diameter. Table 2 gives the mean size for each of the 18 knobs of the northern group and the 13 knobs of the southern group.

The data of table 2 revealed a relationship between knob frequency and knob size that was unexpected though in keeping with the writer's belief that the knob-forming positions that collect knob material most frequently will also collect a larger quantity of knob material. In the southern teosinte group, six of the eight knobs having a frequency of 100 percent are above the mean size, and of the five remaining knobs having frequencies below 100 percent, only one has a knob size above the mean. In the northern teosinte group the condition is similar, since all of the six knobs with a frequency of 100 percent are above the mean knob size, five of the six knobs with frequencies between 85 and 100 percent are above the mean knob size, and the six knobs with low frequencies are all below the mean size.

Since there seems to be an unquestionable relationship between knob frequency and knob size, it was thought that the two might be

combined by summing the measurements for each knob and dividing the total by the number of plants, both with and without knobs. This gave the mean amount of knob material going to each knob-forming position, and from these values the mean amount of knob material present in each plant was calculated. Table 2 gives these means for each knob position of the northern and of the southern teosintes and the mean amount of knob material per plant for each group.

From the data of table 2 it is apparent that in spite of the larger number of positions collecting knob material in teosinte from northern Guatemala, the group is characterized by a lower amount of knob material per plant and by a lower mean knob size than the teosinte from southern Guatemala.

If the 20 arms of the 10 chromosomes of teosinte from southern Guatemala are examined for the supposed relationship between knob position and knob frequency, it will be noticed that the 2 longest arms and the 4 shortest arms have no knobs; also that there is a slight reduction in knob frequency for the knobs terminating the third, fourth, and fifth longest arms. This suggests that the regions terminating the medium-length chromosome arms of teosinte from southern Guatemala are more favorably located for forming knobs than the regions terminating long and short arms.

Knob frequency in this group of teosinte seems to be determined by the length of the chromosome arms. In the pollen mother cells of this group, as in corn, the shorter chromosome arms seem too short and the longer too long to bear terminal knobs.

The relationship between knob position and knob frequency in corn (?) was shown by a curve that cut each arm at the region considered to be most favorable for knob formation. It seems unnecessary to use such a complicated curve to mark the position most favorable for knob formation in teosinte of southern Guatemala when the data on knob frequency are only sufficient to indicate that the most favorable position is beyond the end of the shorter chromosomes and is internal for the longer chromosomes.

The curve (straight line) that cuts each arm at its most favorable knob position and seems to answer the requirements of the data available is one that cuts the terminal point of an arm  $25\mu$  long. The most favorable knob positions on arms longer and shorter than  $25\mu$  were found by determining the difference between  $25\mu$  and the length of each arm. Then one-third of this difference was added to each arm if the arm was shorter or subtracted if the arm was longer than  $25\mu$ . By this empirical method the distance from the fiber attachment of the point most favorable for knob formation was found on each arm or arms produced.

Such a curve gives an increasing ratio between the distance from the fiber attachment and the most favorable knob-forming position and between  $25\mu$  and the distance from the most favorable knob position to the end of the chromosome when the chromosomes are longer and shorter than  $25\mu$ . It also gives comparable ratios to chromosomes too short or too long to bear terminal knobs.

Figure 1 shows the 20 chromosome arms of the southern teosinte group arranged in order of their length. On this figure are drawn two curves: (1) The dotted line at a position approximately that

used to mark the most favorable position for knob formation on the arms of corn chromosomes; and (2) the unbroken line, which is the curve thought to mark the most favorable knob position on each teosinte arm. A study of the points marked on each arm by these two curves will show that the one used in corn places the terminal region of most of the shorter arms and two of the longer arms of the southern teosinte group in a more favorable position for knob formation than the terminal region of the medium-length arms. On the other hand, the curve considered typical for teosinte (fig. 1) changes the relationship between the knobs and the most favorable knob-forming points so that the terminal regions of the medium-length chromosome arms

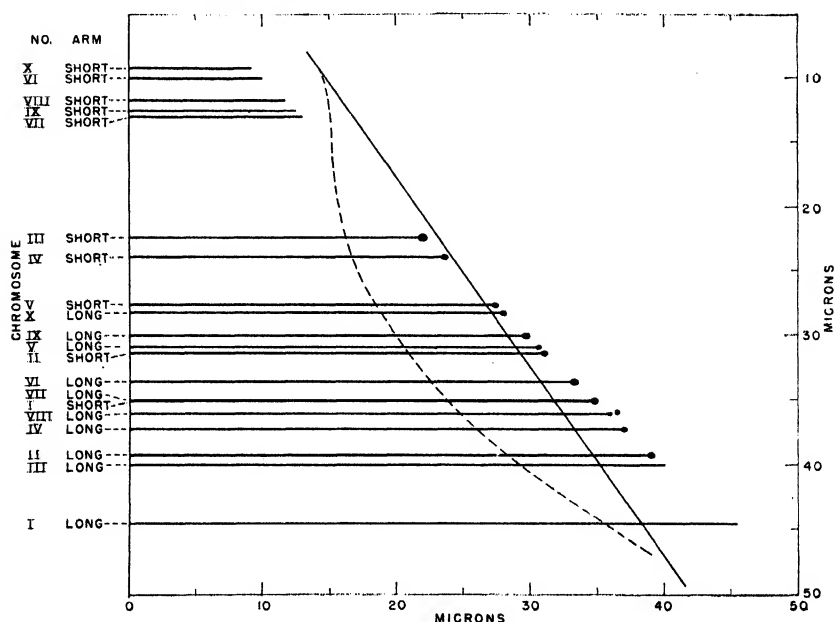


FIGURE 1.—Diagram of the 20 chromosome arms of southern Guatemalan varieties of teosinte, showing the curve that cuts each arm at the most favorable knob-forming position and the curve (broken) that cuts each arm at the most favorable knob-forming position for arms of corn chromosomes of these lengths.

are more favorably located for knob formation than are the terminal regions on the long and short arms.

Since the data discussed above indicate that the most favorable position for knob formation is farther removed from the fiber attachment in teosinte than in corn, it is thought the gradient along the chromosome arms of the southern teosinte group is different from that controlling knob formation on corn chromosomes. This change has resulted in reducing the possibility of finding knobs terminating the short arms of this group of teosintes and has put the terminal point of all medium-length arms in a much more favorable knob-forming position.

Turning again to table 2 and comparing the mean knob sizes for the two groups of teosinte, especially the values given when size and frequency have been combined, it will be noticed that some of the

larger knobs found in northern teosinte chromosomes are on arms that were without knobs in the southern teosinte group, while in other instances some of the smaller knobs are on arms that were terminated by large knobs in the southern group.

Selecting from table 3 all chromosome arms of the northern group with lengths between approximately  $25\mu$  and  $35\mu$ , it will be found that five of these have mean knob sizes near or above the average mean size and that four are appreciably below. This difference in knob size on arms of nearly the same length seems to assume significance when it is realized that the former belong to the longer chromosomes while the latter belong to the shorter chromosomes. This separation of the four shorter chromosomes from the longer chromosomes led

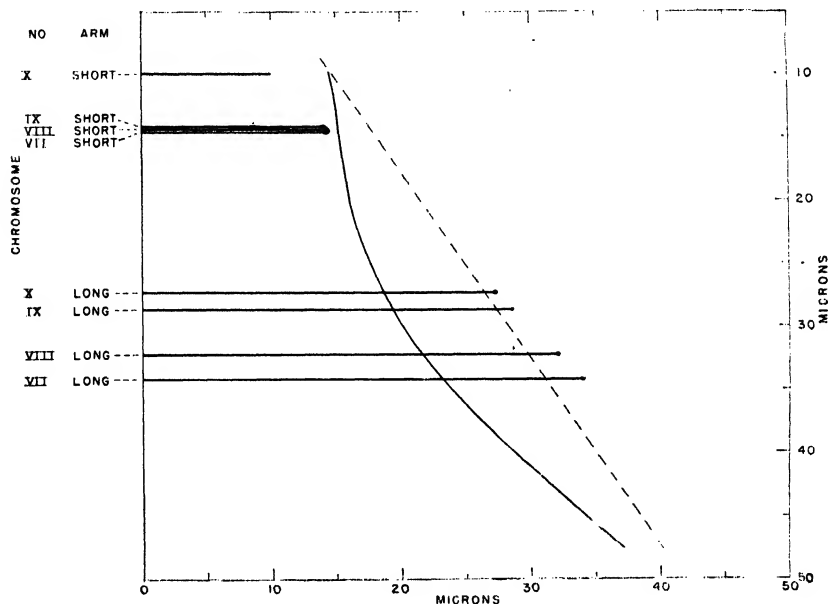


FIGURE 2.—Diagram of the eight chromosome arms of the four shortest chromosomes of northern Guatemalan teosinte, showing the curve that cuts each arm at the most favorable knob-forming position and the curve (broken) that cuts each arm at the most favorable knob-forming position for arms of southern Guatemalan teosinte chromosomes of these lengths.

to an inspection of the knobs on the short arms of these chromosomes. In the southern teosinte group these four arms were without knobs, but in the northern group, three of the four arms, instead of being without knobs, are terminated by large knobs.

Table 3 shows that these four arms in the northern group are not very different in length from the corresponding arms in the southern group. It would seem then that a different factor controls knob formation on the four shortest chromosomes of the northern teosinte group from that which controls knob formation on the chromosomes of the southern group.

By plotting the eight arms of the four shortest chromosomes of the northern Guatemalan teosinte (fig. 2) and superimposing the two curves



of figure 1, it is apparent that the terminal points of each arm, if controlled by the corn gradient, will not have the same ability to form knobs, and that it will be the shortest and the longest arms that will be without knobs or will have only small knobs. If the southern teosinte gradient were controlling the knob formation, it would be the longer arms that would be terminated by the largest knobs. The data on size of knobs clearly point to the presence of a gradient similar to that found in corn for the four shortest chromosomes of the northern teosinte group.

By plotting the 12 arms of the 6 longest chromosomes of the northern Guatemalan group (fig. 3) and superimposing two curves of figure 1, it is apparent that a gradient such as exists in the southern

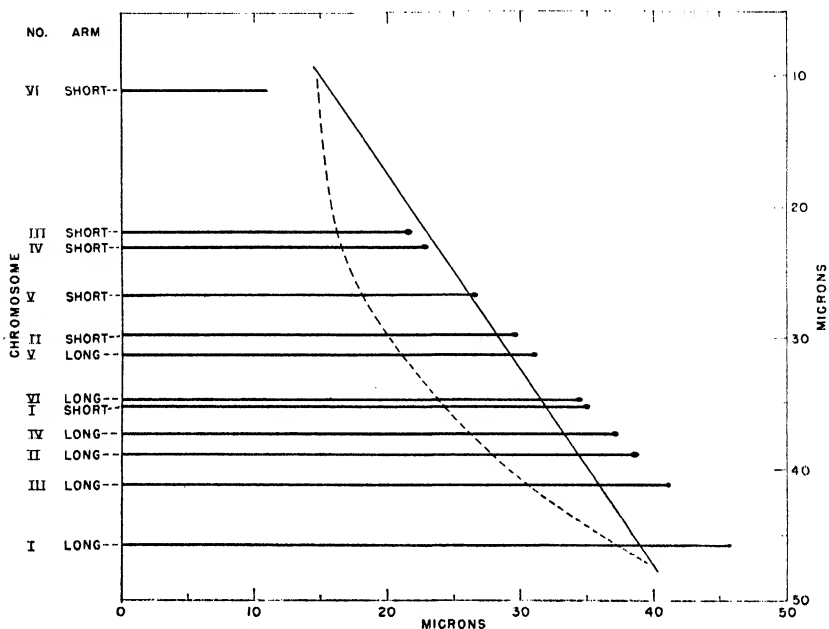


FIGURE 3.—Diagram of the 12 chromosome arms of the 6 longest chromosomes of northern Guatemalan teosinte, showing the curve that cuts each arm at the most favorable knob-forming position and the curve (broken) that cuts each arm at the most favorable knob-forming position for arms of corn chromosomes of these lengths.

teosinte group puts the terminal point of all but the longer arms in a favorable knob-forming position, while a gradient such as exists in corn puts the terminal points of the medium-length arms in the most unfavorable knob-forming position. The size and frequencies of the knobs on the arms of the 6 longest chromosomes of the northern group clearly indicate that knob formation is controlled by a gradient similar to that characteristic of the southern teosinte group.

If some of the northern teosinte chromosomes are controlled by one gradient and others by another, this would account for the fact that more knob-forming points are favorably located to collect knob material in this group than in the southern group. Therefore it is not alone the amount of the knob material available that determines

the number of knobs present but also the number of favorably located knob-forming points combined with the amount of knob material.

It is this comparison of the number, size, and position of the knobs of the two teosintes with each other and with corn that has led to the conclusion that the chromosomes of northern teosinte are more like the chromosomes of corn than are those of southern teosinte.

#### DISCUSSION AND CONCLUSIONS

The result of this comparison of the chromosomes of teosinte from southern Guatemala with those of northern Guatemala has led to the conclusion that the difference in length is so slight and the position of the fiber attachment is so nearly the same that there is no reason to believe that the homologues of the two groups are significantly different in these morphological characters.

The position and number of knobs of the northern group have been shown to be sufficiently different, however, from those of the southern group to distinguish readily plants of either group by the appearance of their chromosomes. This difference is most noticeable in the 4 shortest chromosomes and is thought to be due to a change in the factor or factors controlling chromosome gradient. Such a change is thought to give the 4 shorter chromosomes of the northern teosinte a gradient quite different from that of its 6 longest chromosomes and from that of all the chromosomes of the southern teosinte. The effect of the two gradients on the 10 chromosomes of the northern teosinte has been to put 18 knob-forming points into positions favorable for collecting knob material. Consequently, northern teosinte chromosomes have more knobs than southern teosinte, in spite of the fact that there is less knob material available in the pollen mother cells of the former than there is in the latter group.

Another possible difference in the homologous chromosomes of the two teosinte groups is in the linear arrangement of the genes. The writer has found in some recent unfinished studies that from one to four chromosome bridges are not unusual in pollen mother cells of the first division anaphases of  $F_1$  Florida teosinte-corn hybrids, while O'Mara (9) finds constant and regular pairing between chromosomes of corn and those of teosinte from Nojoyá.

As in the comparison of the chromosomes of the two teosinte groups, so in the comparison between teosinte and corn chromosomes it is the position of the knobs that causes the greatest visible difference. The knobs on corn chromosomes are found much nearer the fiber attachment than the knobs on teosinte from southern Guatemala, and only in the four short chromosomes of teosinte from northern Guatemala does the position of the knobs suggest that their formation is controlled by the same factor or factors as in corn. These four chromosomes are the only ones with a gradient comparable to that of corn chromosomes.

Thus it may be concluded from this comparative study of the number and position of chromosome knobs that it is a difference in chromosome gradient which has made some of the chromosomes of the northern teosinte group appear more like their homologues of corn than like their homologues in the southern group.

The conclusions from this comparison of chromosome length of the two teosinte groups give no indication of a real difference in length of

the homologues in teosinte. Even the difference between teosinte and corn, previously suggested by the writer (5), may be due entirely to a physical lengthening of a chromosome by the presence of a large knob at a considerable distance from the fiber attachment.

It is thought, therefore, that, excluding the few inversions observed in crosses between teosinte from southern Guatemala and corn, a change of even a single-gradient controlling factor might suffice to change all the chromosomes of a teosinte from the southern group to approximately those of the northern group or even to those of corn or vice versa.

The similarity and difference between the chromosomes of teosinte and corn have been the subject of intensive study by several investigators (1, 2, 3). Such studies have been carried out from the point of view of the geneticist, the cytologist, and the cytogeneticist.

The outcome of these studies has been well summarized in the mind of the writer by the following statement of Arnason (1): "The results suggest that gene changes rather than changes in the gross structure of the chromosomes may have been chiefly responsible for the difference of maize and annual teosinte."

If this comparison between teosinte chromosomes from different geographical regions and between the chromosomes of teosinte and corn be extended to include the annual teosintes of Mexico, it will be found that the chromosomes of the Mexican teosintes are more cornlike than the chromosomes of teosintes from Guatemala. Some of the investigators of the chromosomes of Mexican teosintes have failed, however, to recognize that according to competent judges these teosintes are admixtures of teosinte and corn. The writer also would like to consider that Mexican teosintes have not been contaminated with corn and thus have available for comparison a graded series from the least cornlike types of southern Guatemala to the most cornlike types from Mexico. Unquestionably, such a series of teosintes no longer exists in isolated regions that have kept the teosintes free from corn contamination, but it does not seem unreasonable to the writer that, with a common ancestral type in which a few major mutations have occurred, such a graded series in teosinte might have existed at some earlier period.

*Euchlaena perennis* Hitchc., a perennial teosinte that has been almost overlooked in a recent publication (8) on the origin of corn and its relatives, seems to deserve serious consideration in any discussion of the relationship between teosinte and corn. Unfortunately, this species is known only in its tetraploid form. Even in this form it seems to show that true teosintes may be no further removed from corn than they are from each other. A comparison of the prophases of the  $F_1$  of *E. perennis*  $\times$  corn with those of the  $F_1$  of *E. perennis*  $\times$  southern Guatemalan teosinte shows that there is a partial synapsis between the teosinte and corn chromosomes in the former hybrid, while in the latter there is little or no synapsis between the chromosomes of the two teosintes.

The position and number of chromosome knobs have proved useful in distinguishing the two groups of teosintes from Guatemala. The almost complete absence of knobs on perennial teosinte (5) clearly places this teosinte in a class by itself.

The amount of knob material in the pollen mother cells in the teosintes from three isolated localities shows that knob material is most

abundant from teosintes of the southern region and is reduced to practically zero in perennial teosinte from central Mexico. A change in geographical location of a few hundred miles has affected the knob material in teosintes in much the same manner as a change from Mexico and Central America to localities both north (6) and south has reduced the amount of knob material in the pollen mother cells of corn.

#### SUMMARY

In gross morphological characters, such as chromosome length and position of fiber attachments, the chromosomes of teosinte are very similar to each other and to their homologues in corn.

The position and number of the chromosome knobs of teosinte have characteristic differences that make teosintes from different localities reasonably distinguishable.

These distinguishing features are of the type that indicates that teosintes differ in their relationship to each other and to corn, the teosintes of northern Guatemala being slightly more cornlike than those from southern Guatemala, while perennial teosinte from Mexico is thought to have chromosomes even more similar to those of corn.

Considering only the morphological characters of the chromosomes of teosinte and corn, it seems possible that a few gene mutations in the original type of teosinte may have given rise to a form that could have become the progenitor of corn.

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# STUDIES ON THE PREPARATION OF MUSHROOM COMPOST<sup>1</sup>

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## INTRODUCTION

In a previous paper<sup>2</sup> it was pointed out that there is a well-defined tendency for mushroom compost heaps to become stratified so that manure in various parts of a heap is composting under different conditions of temperature, moisture, and aeration. Undoubtedly these differences lead to diversities in the rate of decomposition, type of microbial flora, and chemical changes in separate areas of the heap.<sup>3 4 5 6</sup> This more or less unavoidable heterogeneity in composting conditions causes a variability in the finished compost that makes experimental yield tests with mushrooms difficult and is an important source of variability in commercial yields.

The stratification of mushroom compost heaps into patterns with regard to physical conditions is dependent largely on the size, shape, and compactness of the heap. These patterns also change somewhat with the progress of the fermentation. The same principles, however, are basic to all heaps and may perhaps be best illustrated by a detailed description of the physical conditions in an average compost heap.

The pattern most commonly encountered is illustrated in figure 1. Usually four areas can be distinguished, having fairly distinct sets of physical conditions. The first (area *A*) is an outside layer from 2 to 6 inches thick, which varies in temperature from that of the surrounding air to 110° F. Compost in this area has a tendency to become dry on the sides of the heap, owing to excessive aeration; and in cool or wet weather it becomes wet on the top, owing to condensation or precipitation. The second layer (area *B*) lies just inside the first. It is well aerated, is moderately moist, and varies in temperature from 110° to 140°. The third (area *C*) extends around the inside of the heap like a huge doughnut from 2 to 4 feet from the sides and 1 to 3 feet from the top of the heap. This area contains the warmest compost in the heap, ranging from 150° to 180°. It is probable that the high temperatures are attained in this area because the compost in it is well insulated and receives sufficient air for oxidation without an excess to cause loss of heat by convection. The fourth distinct

<sup>1</sup> Received for publication December 31, 1940.

<sup>2</sup> LAMBERT, EDMUND B., and DAVIS, A. C. DISTRIBUTION OF OXYGEN AND CARBON DIOXIDE IN MUSHROOM COMPOST HEAPS AS AFFECTING MICROBIAL THERMOGENESIS, ACIDITY, AND MOISTURE THEREIN. *Jour. Agr. Res.* 48: 587-601 illus. 1934.

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<sup>5</sup> ———, UMBRIET, W. W., and CORDON, T. C. THERMOPHILIC ACTINOMYCETES AND FUNGI IN SOILS AND IN COMPOSTS. *Soil Sci.* 47: 37-61, illus. 1939.

<sup>6</sup> JENKINS, S. H. ORGANIC MANURES. *Imp. Bur. Soil Sci. Tech. Commun.* 33, 64 pp., illus. 1935.

region (area *D*) occupies the entire lower central part of the heap. The oxygen in this area is used up within 7 or 8 hours after the heap is "turned," so that the material is usually decomposing under anaerobic conditions.

To obtain a comparatively uniform compost, mushroom growers mix the manure from different parts of the heap at weekly intervals.

In spite of this mixing, however, there are noticeable dissimilarities in the appearance of the compost in the different areas, as indicated in figure 1, which persist during most of the composting process. For example, during the first and second turnings the manure in the anaerobic region (area *D*) is noticeably less decomposed than the

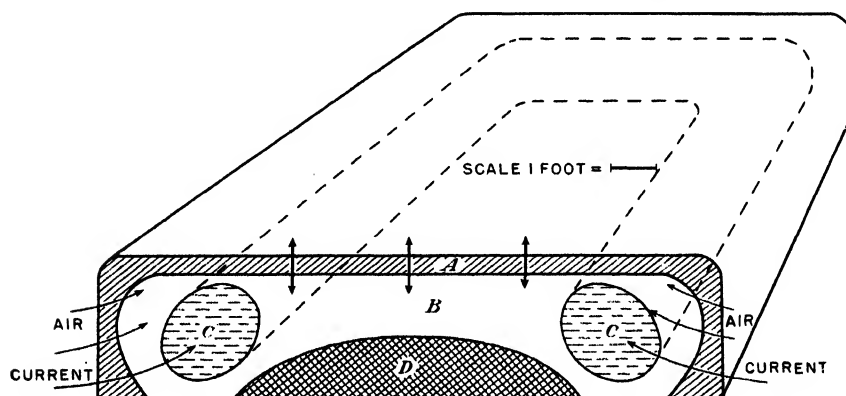


FIGURE 1.—Diagrammatic section through a mushroom compost heap, showing the usual gross differences in temperature and aeration in four areas: *A*, Aerobic, 110° F. or less; *B*, aerobic, 110° to 140°; *C*, aerobic, 150° to 180°; *D*, anaerobic, 100° to 130°.

remainder of the heap. Moreover, it has an unpleasant putrid odor and an acid reaction, whereas the remainder of the heap is usually alkaline. The compost in the high-temperature region (area *C*) soon attains a characteristic glossy brown color and has a caramel odor quite distinct from that of the manure in the remainder of the heap. In area *B* there is more evidence of fungus and actinomycete growth than in either area *C* or *D*. In the portions of area *B* with a temperature range between 130° and 140° F. the manure becomes speckled with a white spore dust from the extensive development of thermophilic actinomycetes. This actinomycete flora also appears in the beds during "sweating out," when similar conditions of aeration and temperature are encountered.

These characteristic differences in the appearance of the compost in various parts of mushroom compost heaps suggest differences in suitability for the growth of mushroom mycelium. The studies outlined in this paper were conducted primarily to learn whether there are demonstrable differences in this respect, (1) when conditions in the different areas are considered separately, and (2) when these conditions are considered in different combinations and sequences. Experiments are also described that were made to determine the effect of different controlled composting conditions on the rate of decomposition of the manure, and on the rate at which fresh manure is converted to compost suitable for the growth of mushroom mycelium.

**EFFECT OF CONDITIONS IN DIFFERENT AREAS IN THE COMPOST HEAP ON SUITABILITY OF COMPOST FOR MUSHROOM MYCELIUM**

The most direct method of studying the effect of conditions in different areas of the compost heap appeared to be the sampling of the various areas during successive stages of the composting process and the inoculation of the samples with mushroom spawn.

For purposes of comparison, triplicate samples of manure were taken separately from areas *B*, *C*, and *D* of a 20-ton compost heap at the time of mixing or "turning." Since the compost heap was



FIGURE 2.—Samples of manure incubated for 16 days under controlled conditions: *A*, 130° F., partly aerated; *B*, 170°, aerated; *C*, 130°, aerated. During incubation the jars were laid in a horizontal position to provide drainage, and the cotton plugs were removed for better aeration.

turned at weekly intervals, the manure had been subjected to composting conditions characteristic of the area sampled for at least 1 week before sampling. After the first week the manure in each area had been mixed with manure from other areas by one less turning than the number of weeks indicated in table 1. These samples were adjusted to about 160-percent moisture on a dry-weight basis, placed in quart jars, as shown in figure 2, and incubated in a horizontal position at 130° F. for 6 hours to kill insects. They were then cooled to 70° and inoculated with mushroom spawn.

A comparison of the growth of mushroom mycelium and of weed molds in these inoculated samples is shown in table 1. The samples



from area *B* (fig. 1) were well suited for the growth of mushroom spawn. The samples from areas *C* and *D*, however, were frequently overgrown with weed molds such as *Chaetomium*, *Coprinus*, *Trichoderma*, *Monilia*, and *Oedocephalum*. As a result, the growth of the mushroom mycelium was retarded or checked entirely. This type of experiment was repeated several times with essentially the same results.

TABLE 1.—Comparative growth of mushroom spawn and weed molds in samples taken from different parts of a standard mushroom compost heap during successive turnings, when subjected to a 6-hour period of "sweating out" at 130° F., aerated, and subsequently spawned and incubated at 70°

Area of compost heap sampled (fig. 1)	Outdoor-composting period	Average pH value at time of spawning	Growth <sup>1</sup> of mushroom spawn	Growth <sup>1</sup> of weed molds				
				<i>Coprinus</i>	<i>Trichoderma</i>	<i>Monilia</i>	<i>Chaetomium</i>	<i>Oedocephalum</i>
	Weeks							
<i>B</i>	1	8.1	++++	+	0	0	0	0
	2	7.8	++++	0	0	0	0	0
	3	7.7	++++	0	0	+++	0	0
	4	7.6	++++	0	0	0	0	0
<i>C</i>	1	6.8	+	+++	0	+++	0	0
	2	6.7	++++	0	++	0	++	++
	3	8.2	+++	0	0	++	0	0
	4	7.9	++	0	0	+++	0	0
<i>D</i>	1	8.2	++	++	+++	0	0	0
	2	8.7	++	+++	0	++	++	0
	3	8.6	++	+++	0	0	+++	0
	4	8.0	+++	0	0	++	0	0

<sup>1</sup> +++++=excellent; +++=good; ++=medium; +=poor; 0=no growth.

### ALTERNATING COMPOSTING CONDITIONS

The fact that samples taken from area *B* were favorable for mushroom mycelium during the second and subsequent turnings, in spite of a probable admixture of compost formerly in areas *C* and *D*, suggested that conditions in area *B* tend to correct the harmful effects of anaerobic composting or of composting at temperatures over 150° F. To test this hypothesis, a series of samples was selected and prepared in the same manner as previously described except that before spawning the samples were composted in an incubator at 130° for 8 days instead of 6 hours.

In this experiment, as shown in table 2, nearly all the samples were well suited for the subsequent growth of mushroom mycelium and, with the exception of *Chaetomium* in a few samples, the weed molds were eliminated.

It was apparent that aerobic fermentation at 130° F. not only was favorable for subsequent growth of mushroom mycelium but had a beneficial effect on the compost that previously had been made unfavorable by anaerobic fermentation or by overheating. Other experiments were made in which it was repeatedly shown that, conversely, compost favorable for mushroom mycelium becomes unfavorable when incubated in glass jars for 1 or 2 days at an excessively high temperature or when incubated under anaerobic conditions even at a favorable temperature (130°). The injurious effects of overheating seemed to begin at about 150°. Compost so treated could again be made suitable by subjecting it to aerated fermentation for several days at 130°.

TABLE 2.—Comparative growth of mushroom spawn and weed molds in samples taken from different parts of a standard mushroom compost heap during successive turnings, when subjected to 8 days of "sweating out" at 130° F., aerated, and subsequently spawned and incubated at 70°

Area of compost heap sampled (fig. 1)	Outdoor-composting period	Average pH value at time of spawning	Growth of mushroom spawn	Growth <sup>1</sup> of weed molds				
				<i>Coprinus</i>	<i>Trichoderma</i>	<i>Monilia</i>	<i>Chaetomium</i>	<i>Oedocephalum</i>
	Weeks							
B	1	8.1	++++	0	0	0	0	0
	2	7.9	++++	0	0	0	0	0
	3	8.0	++++	0	0	0	0	0
	4	7.8	++++	0	0	0	0	0
C	1	7.5	+	0	0	0	+++	0
	2	7.8	++++	0	0	0	0	0
	3	7.6	++++	0	0	0	0	0
	4	7.9	++++	0	0	0	0	0
D	1	8.4	++++	0	0	0	+	0
	2	8.4	++++	0	0	0	+++	0
	3	8.0	++++	0	0	0	0	0
	4	7.7	++++	0	0	0	0	0

<sup>1</sup> ++++ = excellent; +++ = good; ++ = medium; + = poor; 0 = no growth.

As a whole, these experiments indicate that if manure is first subjected to one set of composting conditions and then changed over to another it soon begins to assume characteristics typical of compost held in the new environment. This is probably explainable on the basis of the new environment's favoring a new fermentation flora, which in turn alters the composition of the compost so as to pave the way either for the mushroom mycelium or for weed molds as the case may be. The favorable flora in the case of the compost fermented under aerobic conditions at 130° F. appeared to be composed largely of thermophilic actinomycetes.

#### RATE OF DECOMPOSITION

Another point of interest relating to the different "areas" in a compost heap is the effect of aeration and temperature on the rate of decomposition of the manure. Numerous investigations have shown that organic matter decomposes more slowly under anaerobic than under aerobic conditions.<sup>7</sup> Recently Waksman, Cordon, and Hulpoi<sup>8</sup> have shown that stable manure compost incubated at 167° F. also decomposes much more slowly than at lower temperatures (84°, 122°, and 150°).

In order to obtain further information on this question, a series of 100 samples of fresh stable manure was incubated in glass jars (fig. 2) under different controlled conditions of temperature and aeration. At intervals of 5 days 2 jars were removed from each group of samples for dry-weight determination. The results of aerobic composting are given in figure 3. At temperatures ranging from 110° to 150° F. nearly half of the dry weight was lost during the first 20 days. At 170° there was very little loss. Conditions typical of area D (fig. 1) in compost heaps were simulated in a series of samples of manure composted in sealed jars which were incubated at 130°. This series lost less than 10 percent of its dry weight over a 30-day period.

<sup>7</sup> See footnote 3.

<sup>8</sup> See footnote 4.

The results of these experiments are in complete accord with earlier work in showing that both anaerobic conditions and temperatures approaching 170° F. are unfavorable for rapid decomposition of stable manure.

#### PREPARATION OF MUSHROOM COMPOST UNDER CONTROLLED FERMENTATION CONDITIONS

In the foregoing experiments, at the end of 2 weeks the manure held at 110° and 130° F. under aerated conditions appeared to be nearly as well decomposed as compost in the average mushroom bed. This suggested that fresh manure could be converted into compost suitable for mushroom mycelium in a comparatively short time under

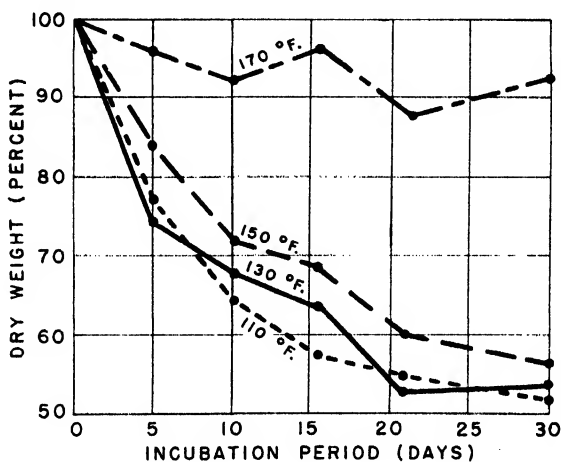


FIGURE 3.—Relation of temperature to rate of decomposition of stable manure under moist aerobic conditions.

favorable conditions. The excellent run of spawn that developed in some of the samples taken from area *B* after 1 week of outdoor composting, as shown in table 1, was further evidence pointing in this direction. Following up these leads, experiments were planned to learn something of the rate at which fresh horse manure could be converted to suitable mushroom compost under different controlled composting conditions.

In one series a comparison was made between manure alone, a mixture of manure and one-third soil by weight, and a mixture of manure and 2 percent of superphosphate. In all cases the supplementary material was thoroughly mixed with the manure, which was chopped into pieces less than 1 inch long and moistened to contain about 200 percent of water on a dry-weight basis. The type of jar used has already been illustrated (fig. 2). Moisture was maintained by inspection and rewatering every 3 days. Temperature was controlled within a 3-degree limit of variation at 110°, 120°, and 130° F. After fermentation periods of 9 days and 11 days, the bottles were removed from the incubator, cooled to room temperature, and spawned with mushroom mycelium.

A satisfactory growth of spawn was obtained in compost prepared at all three temperatures for both 9 days and 11 days. Spawn growth was dense and complete in the bottles containing the supplementary materials, but the manure alone became too wet and failed to support a good growth of spawn.

Another series of bottles containing a mixture of two-thirds manure, one-third soil, and 2 percent superphosphate was composted at 130° F. for 4 days, 6 days, 8 days, and 10 days before spawning. Mushroom mycelium developed satisfactorily in the manure composted 8 days and 10 days, but the samples composted for only 4 days and 6 days were soon overgrown with weed molds.

It was apparent from these experiments that under favorable fermentation conditions fresh manure can be converted into compost suitable for mushroom mycelium in about one-third the time required in the conventional outdoor compost heap. Subsequent experiments, not yet reported in detail, showed that compost prepared in this manner also is capable of producing a normal yield of mushrooms.

#### DISCUSSION AND CONCLUSIONS

It is apparent from the foregoing experiments that the usual method of composting manure for mushroom culture in outdoor heaps is not an efficient procedure. Only a limited portion of the heap, area *B* and parts of area *A* (fig. 1), is suitable for rapid decomposition and conversion of fresh manure to a satisfactory mushroom compost. On the other hand, a considerable portion of the conventional heap, areas *C* and *D*, is known to be decomposing at a comparatively slow rate and frequently to be in a condition favorable for subsequent growth of "weed molds" and unfavorable for mushroom mycelium. This is especially true during the first 2 weeks of composting, when all of the compost in the anaerobic and high-temperature areas is more or less unfavorable for mushroom mycelium.

As the composting proceeds the unfavorable conditions are less in evidence; area *C* becomes less extensive, owing to the disappearance of easily oxidized carbonaceous material, and the compost in area *D* becomes less acid with each turning. During outdoor composting a large part of the beneficial effect of continued composting probably comes from subjecting more and more of the compost to fermentation conditions in area *B* as a result of forking over and mixing the manure. In most cases the final conversion of unfavorable compost to a condition favorable to mushroom mycelium undoubtedly takes place during the "sweating out" process in the mushroom bed.

The sweating-out period is the only part of the composting process during which nearly all of the manure is fermenting under favorable conditions. Since at this time the compost is undergoing aerobic fermentation with a moisture content of 150 to 200 percent on a dry-weight basis and within a temperature range between 120° and 140° F., one can hardly escape the conclusion that the sweating-out process should be considered as an integral part of the composting procedure and not merely as a means of assisting in the eradication of insects and disease fungi. Outdoor composting might well be considered merely as a means of mixing and moistening the manure while carrying it through the initial explosive fermentation preparatory to a final favorable fermentation during sweating out.

Interesting practical considerations are suggested by this viewpoint and by the fact that suitable mushroom compost can be prepared from fresh manure in less than 2 weeks under conditions usually prevailing during sweating out. Small-scale experiments suggest that the outdoor composting period may be materially shortened or eliminated altogether provided sweating out is prolonged at the rate of 1 additional day of sweating out for every 3 days omitted from the usual outdoor composting. The extent to which it would be feasible or desirable to thus modify composting procedure in commercial practice will depend on the feasibility of controlling moisture and temperature during prolonged sweating out and on the prospects for saving labor or manure without reducing the yield of mushrooms.

#### SUMMARY

Consideration is given to the effect of physical factors, principally temperature and aeration, during composting on the subsequent growth of mushroom mycelium in the compost.

The suitability of compost for the growth of mushroom mycelium was determined by inoculation of selected samples with mushroom spawn and incubation at 70° F. in competition with the weed molds present in the compost.

Under aerobic conditions fermentation at temperatures between 120° and 140° F. for 8 to 10 days produced suitable compost. Temperatures over 150° for a few hours rendered the compost unsuitable. Anaerobic fermentation also produced unsuitable compost, even at favorable temperatures.

When manure was composted under different combinations and sequences of these conditions it tended to assume characteristics typical of the last environment to which it was subjected. Thus, aerobic fermentation at moderately high temperatures would again render compost suitable even though it had previously been made unsuitable by excessively high temperature fermentation or by anaerobic conditions.

In commercial mushroom growing, sweating out must be considered an integral part of the composting process. Close control of composting conditions is more important during sweating out than during outdoor composting, since conditions prevailing during sweating out constitute the last composting environment to which the manure is subjected before spawning.

# USE OF LEMNA FOR NUTRITION STUDIES ON GREEN PLANTS<sup>1</sup>

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## INTRODUCTION

The use of *Lemna minor* L. in a micromethod for study of the nutritional requirements of green plants has several obvious advantages. Equipment may be on a very small scale and selected for precision control of environmental factors at a small fraction of the initial cost and maintenance expense required for plants of ordinary size. Duration of experiments may be decreased to 14 days. Growth under aseptic conditions is entirely practicable and easily obtained, thus eliminating all possibility of erroneous results due to the presence of extraneous organisms. Precision control of environment should permit exact quantitative work capable of duplication elsewhere and in the future. Results obtained through the use of a micromethod may be directly checked with different crop plants, with consequent savings in the time and cost associated with large-scale exploratory work ordinarily necessary.

Trace-element studies with a green plant possess an obvious advantage over those with fungi. The former utilize carbon as carbon dioxide, which may easily be supplied free of trace-element impurities. The latter require a supply of sugar. Since a culture of *Aspergillus niger* Van Tiegh. contains 2.5 gm. of sucrose and 0.125 gm. of inorganic salts, it is evident that elimination of impurities in the carbon source is a very important consideration. The purest sugar obtainable contained 0.00087 percent of ash, or the equivalent of 435 parts per billion of impurities in the nutrient solution. This quantity compares with the 790 parts per billion whose addition is required for maximum growth of *Aspergillus*.

*Lemna* has been grown on synthetic nutrient solutions by many investigators (2, 3)<sup>2</sup> and its mineral requirements shown to be quite similar to those of other green plants. The elements known to be required for its nutrition include carbon, hydrogen, oxygen, nitrogen, phosphorus, potassium, magnesium, sulfur, calcium, iron, and manganese (3). No positive evidence has been obtained, however, respecting its need for zinc, copper (4), molybdenum (1), gallium (7), and boron.

Aside from establishing conditions permitting maximum growth, and determining the accuracy in duplication of data, the ascertainment of the trace-element requirements of *Lemna* seemed most important. The higher plants require iron, zinc, copper, manganese, molybdenum, and boron, whereas *Lemna*, as mentioned, is only known to require iron and manganese. It was necessary, therefore,

<sup>1</sup> Received for publication November 4, 1940.

<sup>2</sup> Italic numbers in parentheses refer to Literature Cited, p. 430.

to adapt the method of nutrient-solution purification with calcium carbonate, employed with fungi (5, 6) for the removal of trace-element impurities, to work with *Lemna*. Arnon and Stout (1, 8) applied this technique to the purification of the individual salts of the nutrient solution in establishing the necessity for molybdenum by the tomato plant. Purification of the nutrient solution as a whole, in the manner employed with fungi, has certain advantages, particularly with a plant like *Lemna*, and was therefore used in this investigation. It was also believed that the use of nutrient-solution purification with *Lemna* would permit a closer comparison of the trace-element requirements of these two types of plants.

#### MATERIAL AND METHODS

An aseptic culture of *Lemna minor* obtained from Dr. A. Saeger, Junior College of Kansas City, Kansas City, Mo., was employed in these studies. The plants were grown on 50-cc. portions of nutrient solution in 200-cc. pyrex Erlenmeyer flasks provided with loose cotton plugs to maintain aseptic conditions. Reagent chemicals and water redistilled in a quartz still were used in the preparation of the nutrient solution. The flasks were sterilized after filling at 15 pounds pressure for 20 minutes. Transplantation of plantlets from the stock culture to the experimental flasks was with a platinum loop, employing the standard bacteriological technique to prevent contamination. For this purpose plantlets of minimum size having short roots were selected whenever possible. When harvested they were filtered off by means of a fritted-glass crucible (1 G 3), dried for 3 to 4 hours at 100 to 105° C., and weighed with the analytical balance when cool.

The plants were grown under different conditions. Initially an installation providing continuous light of 500 foot-candles with tungsten lamps (400 watts total) and a temperature of  $25 \pm 1^\circ$  C. was used. The growth period was 2 weeks. In this equipment the light was filtered through a 1-inch layer of water to minimize heating, and the cabinet was ventilated by means of a stream of chilled compressed air. When it was too hot to employ this installation, the flasks were placed in a large north window for 30 days. The illumination varied between 100 to 200 foot-candles. Later an electric refrigerator (9.2 cubic feet) having a special thermostat was provided with 500 foot-candles of white fluorescent light (100 watts total). Temperature in this cabinet averaged  $25 \pm 0.5^\circ$  C. Growth appeared normal under these conditions (continuous light) for the 2-week duration of an experiment. No ventilation was provided other than that brought about by opening the door of the refrigerator to examine the plants.

Where nutrient-solution purification was employed, calcium carbonate was added to a solution containing an excess of constituents. After steaming for 20 minutes at 100° C., the flask was set aside for 2 days. The solution was vigorously mixed by quick rotations of the flask at irregular intervals during this time. It was then filtered through a fritted-glass crucible (1 G 4) and made up to proper acidity with dilute (1.38 N) hydrochloric acid. The acid was redistilled for this purpose by placing two open pyrex bottles, one filled with concentrated acid, the other with water, in a sealed desiccator.

The purpose of these procedures was to permit the precipitate of dicalcium phosphate formed under these conditions to reach equilibrium, particularly as respects atmospheric carbon dioxide. When

employed with fungi, sufficient residual phosphate remains in solution because of the presence of fairly high concentrations of nitrogen salts and sugar. In the absence of sugar and high salt content, practically no phosphate may be left in the nutrient solution. When in equilibrium with atmospheric carbon dioxide, sufficient phosphate remains in solution to provide for the growth of 100 mg. of dry weight per 50 cc. with *Lemna*. In the case of larger plants requiring still higher quantities of phosphate, sufficient should be brought into solution by bubbling through gaseous carbon dioxide before filtering. Sufficient calcium remains in solution after purification, so that none need be added subsequently to filtration.

#### TRACE-ELEMENT DEFICIENCIES WITH REAGENT CHEMICALS

The experimental data of table 1 were obtained with several considerations in mind. Of these the foremost was to ascertain the precision with which results could be duplicated. Comparison of duplicate experiments 1 and 2 indicate that variations of  $\pm 10$  percent from average may occur in percentage yields in simultaneous experiments. The data of duplicate experiments 3 and 4 show a somewhat lesser variation. In the first pair of duplicate experiments, only the results on molybdenum deficiency differ by not more than  $\pm 2.5$  percent. In the second pair, with about double the salt content, agreement in results to less than  $\pm 5$  percent occurred with deficiencies in iron, manganese, calcium, and boron. It is evident that differences of  $\pm 10$  percent from average cannot be accepted as evidence for deficiency unless consistently reproducible at will.

Individual differences among the plants selected for initial transfer are probably responsible for a part of these variations. These can be minimized, if necessary, by the use of a larger number of plants. An equally important consideration is the proper adjustment of the components of the nutrient solution, since fluctuations in an unsuspected essential element can also result in large fluctuations in growth. Further study will be necessary to improve precision in results, but no serious difficulty is anticipated in attaining this objective.

TABLE 1.—*Growth of Lemna minor for 2 weeks under continuous illumination of 500 foot-candles of tungsten light at 25° C.*

Element omitted <sup>1</sup>	Experiment 1, weaker solution, <sup>2</sup> pH = 4.70					Experiment 2, weaker solution, pH = 4.70					Experiment 3, stronger solution, <sup>3</sup> pH = 4.78				
	Yield	Proportion of maximum yield	Acidity at harvest	Size of plants <sup>4</sup>	Color <sup>5</sup>	Yield	Proportion of maximum yield	Acidity at harvest	Size of plants <sup>4</sup>	Color <sup>5</sup>	Yield	Proportion of maximum yield	Acidity at harvest	Size of plants <sup>4</sup>	Color <sup>5</sup>
None.....	Mg. 21.8	Pct. 100.0	pH 7.19	M.	4	Mg. 24.2	100.0	pH 7.33	M.	4	Mg. 29.0	100.0	pH 7.02	M.	4
Fe.....	7.5	34.4	6.75	M.	3	5.4	22.3	6.45	M.	2	7.3	25.2	6.42	M.	2
Zn.....	26.7	122.5	7.22	M.	4	25.7	106.2	7.20	M.	4	27.0	93.1	6.99	M.	3
Mn.....	2.5	11.5	6.11	S.	2	8.6	35.5	6.71	S.	2	11.2	38.6	6.42	S.	1
Mo.....	23.4	107.3	7.04	M.	4	27.3	112.8	7.25	M.	4	27.4	94.5	6.86	M.	4
Ca.....	5	4.1	5.94	S.	0	5	2.1	5.95	S.	0	6	2.1	5.80	0	0
Ga.....	22.2	101.8	7.15	M.	4	21.9	90.5	7.18	M.	4	27.1	93.4	6.89	M.	4

See footnotes at end of table.



TABLE 1.—Growth of *Lemna minor* for 2 weeks under continuous illumination of 500 foot-candles of tungsten light at 25° C.—Continued

Element omitted <sup>1</sup>	Experiment 4, stronger solution, pH=4.78					Experiment 5, stronger solution with 0.5 percent of sucrose, pH=4.35					Experiment 6, stronger solution with 0.5 percent of sucrose, pH=4.54				
	Yield	Proportion of maximum yield	Acidity at harvest	Size of plants <sup>4</sup>	Color <sup>5</sup>	Yield	Proportion of maximum yield	Acidity at harvest	Size of plants <sup>4</sup>	Color <sup>5</sup>	Yield	Proportion of maximum yield	Acidity at harvest	Size of plants <sup>4</sup>	Color <sup>5</sup>
None.....	Mg.	Pct.	pH	M.		Mg.	Pct.	pH	M.		Mg.	Pct.	pH	M.	
Fe.....	26.8	100.0	7.08	M.	4	74.9	100.0	6.97	M.	4	56.9	100.0	6.65	M.	4
Zn.....	7.8	29.1	6.38	M.	2	31.4	41.9	6.59	M.	2	32.5	57.1	6.41	L.	2
Mn.....	29.2	109.0	6.82	M.	3	74.8	99.9	6.10	M.	4+	54.0	94.9	5.88	M.	5
Mo.....	11.2	41.8	6.37	S.	1	20.0	26.7	5.97	S.	2	.9	1.6	4.62	S.	1
Ga.....	26.9	100.4	7.05	M.	4	61.8	82.5	6.27	M.	4+	50.5	88.8	6.22	M.	4
Ca.....	.6	2.2	5.33	0	0	.7	.9	4.64	0	0	55.8	98.1	6.69	M.	4
B.....	25.2	94.0	6.89	M.	4	70.5	94.1	6.83	M.	4	.2	.4	4.73	0	0
											57.6	101.2	6.57	M.	4

<sup>1</sup> Iron, zinc, manganese, molybdenum, gallium, calcium, and boron at concentrations of 0.15, 0.04, 0.05, 0.02, 5.0, and 0.05 mg. per liter, respectively.

<sup>2</sup> Water, 1,000 cc.; KNO<sub>3</sub>, 0.18 gm.; KH<sub>2</sub>PO<sub>4</sub>, 0.10 gm.; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.04 gm.

<sup>3</sup> Water, 1,000 cc.; KNO<sub>3</sub>, 0.35 gm.; KH<sub>2</sub>PO<sub>4</sub>, 0.20 gm.; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.10 gm.

<sup>4</sup> Tiny, small, medium, and large are indicated by first letter only. Zero indicates that no growth took place, owing to rapid death of the plants.

<sup>5</sup> Color is graded as 0 (white) to 5 (normal green).

There can be little doubt that omission of iron, manganese, and calcium from the nutrient solution may result in decreased growth when reagent chemicals are employed. Use of the higher salt concentration in experiments 3 and 4 tended to diminish the necessity for addition of these trace elements and to increase the uniformity in response.

In the last two duplicate experiments (5 and 6) the nutrient solution contained 5 gm. of sucrose per liter. The large increase in growth brought about through addition of sucrose definitely indicated photosynthesis to be the limiting factor for growth. This result was not unexpected, since Ashby and Oxley (2) had determined 1,600 foot-candles to be optimum for growth of *Lemna*. Miscellaneous experiments with increased carbon dioxide content of the air confirmed the need for additional light intensity. Results on trace-element deficiencies with 0.5 percent of sucrose were somewhat poorer with iron, while addition of molybdenum became necessary. Gallium became nontoxic, though in previous experiments its addition had brought about slightly decreased growth.

#### TRACE-ELEMENT DEFICIENCIES AFTER NUTRIENT-SOLUTION PURIFICATION

Nutrient-solution purification with calcium carbonate led to increased trace-element deficiencies (table 2) with iron, zinc, manganese, molybdenum, gallium, and boron. Sufficient residual calcium remained in the nutrient solution after purification to provide completely for the needs of the plant. Omission of copper from the purified nutrient solution did not decrease growth.

TABLE 2.—*Growth of Lemna minor in solutions purified with calcium carbonate*

Element omitted <sup>1</sup>	100-200 foot-candles of north light during July and August for 30 days						500 foot-candles of continuous white fluorescent light at 25° C. for 2 weeks								
	Purified with CaCO <sub>3</sub> , pH=3.85			Purified with CaCO <sub>3</sub> , pH=5.34			Purified with CaCO <sub>3</sub> , pH=4.60			Purified with CaCO <sub>3</sub> , pH=5.45			Purified with CaCO <sub>3</sub> , pH=5.46		
	Yield	Proportion of max- imum yield	Acidity at harvest	Size of plants <sup>2</sup>	Color <sup>3</sup>	Yield	Proportion of max- imum yield	Acidity at harvest	Size of plants <sup>2</sup>	Color <sup>3</sup>	Yield	Proportion of max- imum yield	Acidity at harvest	Size of plants <sup>2</sup>	Color <sup>3</sup>
None	Mg., 13.5	Pct., 100.0	pH, 5.56	M.	5	Mg., 17.3	Pct., 100.0	pH, 5.94	L.	4	Mg., 19.4	Pct., 100.0	pH, 5.80	L.	4
Fe	6.1	7.4	4.05	M.	4	2.6	15.0	5.42	L.	2	2.2	11.3	5.15	S.	4
Zn	4.5	33.3	4.19	M.	4	8.5	49.1	5.60	L.	2	17.7	91.2	5.48	L.	4
Mn	—	2.2	3.83	T.	1	1.6	9.2	5.28	S.	1	1.4	7.2	4.03	T.	1
Mo	—	4.4	3.79	S.	3	2.3	13.2	5.31	M.	1	11.1	57.2	5.47	L.	4
Ga	17.7	131.1	5.62	M.	3	9.2	53.2	5.71	L.	4	15.8	81.4	5.82	L.	4
B	6.8	50.4	5.06	M.	5	5.0	28.9	5.59	L.	4	14.6	75.3	5.77	L.	4

<sup>1</sup> Iron, zinc, manganese, molybdenum, gallium, and boron at 0.30, 0.04, 0.05, 0.02, 0.02, and 0.04 mg. per liter, respectively.

<sup>2</sup> Water, 1,000 cc.; KNO<sub>3</sub>, 0.50 gm.; KH<sub>2</sub>PO<sub>4</sub>, 0.70 gm.; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.50 gm.; CaCO<sub>3</sub>, 0.30 gm. HCl (1.38 N) added in quantities of 1.3 to 1.0 cc. per liter after filtration.

<sup>3</sup> Plants used in starting experiment much larger than usual.

<sup>4</sup> Plants used in starting experiment much larger than usual. The carbon dioxide content of the air was also increased.

<sup>5</sup> See footnote 4, table 1.

<sup>6</sup> See footnote 5, table 1.

## SYMPTOMS OF TRACE-ELEMENT DEFICIENCY

Symptoms of trace-element deficiency were not specific but usually consisted of a general chlorosis, sometimes greater at the base of the frond and at other times at the apex. Extreme manganese deficiency led to a specific chlorosis characterized by the presence of irregular bleached spots.

Size of fronds usually decreased with deficiency except in the case of zinc. It seems probable that the differences in responses observed are correlated with specific differences in quantitative effect of the different elements and do not differ qualitatively.

## RELATION OF ACIDITY TO RESULTS

Acidity ranging from pH 3.85 to pH 5.46 permitted growth of *Lemna*. This is about the range found favorable for growth of green plants in general. Growth appeared better at acidities less than that corresponding to pH 4.0.

The effect of growth on acidity of the nutrient solution was to cause a decrease. The extent of this decrease corresponded roughly to the extent of growth.

## DISCUSSION

The results obtained with the calcium carbonate method of nutrient-solution purification indicate its applicability to work with green plants. Its use led to a definite improvement in the data on trace-element deficiencies with respect to the extent of the deficiencies and the number of elements needed. The necessity for the addition of iron, zinc, manganese, molybdenum, gallium, and boron to the nutrient solution was demonstrated following calcium carbonate purification.

The difficulties associated with the use of this method of studying the trace-element requirements of plants are due primarily to the complete precipitation of phosphate and possibly of certain of the essential trace elements. An attempt to employ this method before the discovery of the necessity for copper, manganese, molybdenum, gallium, and boron would most likely have resulted in such poor growth that the method would have been discarded. An adequate concentration of phosphate for *Lemna* can be assured by permitting the reaction mixture to reach equilibrium at room temperature before filtration. Though it was not necessary to resort to it with *Lemna*, the use of carbon dioxide at tensions higher than atmospheric should increase solution of the dicalcium phosphate precipitate according to known solubility data.

The results reported for gallium are not considered entirely adequate proof of its essentiality for *Lemna*. Gallium could not be replaced, however, by the other constituents of the nutrient solution, including nitrogen, phosphorus, potassium, magnesium, sulfur, calcium, iron, zinc, manganese, molybdenum, and boron. Neither were the following elements found in preliminary tests able to replace gallium in the nutrient solution: Copper, sodium, iodine, cobalt, lanthanum, scandium, vanadium, aluminum, germanium, tin, lead, titanium, zirconium, cerium, thorium, nickel, and beryllium. However, replacement tests with the elements have not been com-

pleted as yet. It seems quite probable, nevertheless, that gallium will be found essential for growth of *Lemna*, together with iron, zinc, copper, manganese, molybdenum, and boron (7).

An explanation for the improvement in results with boron would appear to require the assumption that only adsorption on the precipitate of dicalcium phosphate is concerned. No chemical reaction is known to the writer whereby boric acid would be precipitated under these conditions.

The results on trace-element requirements of *Lemna* parallel those of *Aspergillus* quite closely, both with respect to the elements that are essential and their effective concentrations. Exceptions exist, of course, since these forms differ widely in morphology and physiology. Boron and calcium are apparently not required by *Aspergillus*, whereas both are essential for *Lemna*. Zinc and copper are necessary for *Lemna*, in far lesser concentration than for *Aspergillus*, apparently. Results with *Lemna* were only fair with zinc, and the need for copper could not be demonstrated.<sup>3</sup>

*Aspergillus* requires iron, zinc, copper, manganese, molybdenum, and gallium in concentrations of 0.30, 0.30, 0.075, 0.075, 0.02, and 0.02 mg. per liter, respectively, after purification of the nutrient solution. *Lemna* required iron, zinc, copper, manganese, molybdenum, gallium, and boron in concentrations of 0.30, 0.04, 0.0, 0.05, 0.02, 0.02, and 0.04 mg. per liter, respectively, after nutrient-solution purification. It is interesting to note that the tomato plant was found by Arnon and Stout (1) to require 0.01 mg. of molybdenum per liter after calcium carbonate purification of the individual salts.

Further improvement in technique with *Lemna* should include modification in experimental conditions so as to use light of optimum intensity. This is about 1,600 foot-candles, as already mentioned. The basis for this statement is readily understood. The quantity of any trace-element impurity can be decreased by purification to an extremely low level, but, unless other conditions for growth are optimum, the poor yield of the control tends to eliminate this advantage. Poor yields of the control also increase the percentage error of duplication. It seems probable, therefore, that results on deficiencies obtained through application of this technique to crop plants should prove superior to those obtained with *Lemna*. *Lemna*, nevertheless, has proved adequate to serve in a micromethod for the study of mineral requirements of green plants.

#### SUMMARY

*Lemna minor* L. was grown aseptically in pyrex flasks with illumination supplied by tungsten lamps, fluorescent lamps, or north light. Deficiencies in iron, manganese, and calcium were found to exist in nutrient solutions prepared with reagent chemicals. Use of the calcium carbonate method of nutrient-solution purification led to a definite intensification of these effects. The addition of iron, zinc, manganese, molybdenum, gallium, and boron was found necessary after purification. The conditions necessary for the use of this method with green plants were established, and the use of the method is suggested in the study of trace-element requirements of crop plants.

<sup>3</sup> Later trials gave positive results with copper at 0.01 mg. per liter.

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# BODY FORM IN GROWING CHICKENS<sup>1</sup>

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## INTRODUCTION

Using adult female chickens fed and managed in a similar manner, Jaap and Thompson (8)<sup>2</sup> demonstrated heritable differences in body shape. The criteria of conformation used were the ratios obtained by dividing length of shank, body depth, and length of keel by the cube root of body weight. Such measures of body conformation in live birds have proved valuable for predicting visual body shape (7) when chickens or turkeys are being prepared for human consumption. The observations reported in earlier papers (7, 8) were made in the latter part of the growth period and at maturity. The present study was planned to determine whether the above measurements may be used to detect heritable differences in body shape of live chickens at an early stage of growth, the so-called "broiler" ages.

A large proportion of market poultry is killed early in the growth period. In order to reserve for breeders those chickens having superior body conformation, it is important to measure body shape and other characteristics of market quality as early as 8 weeks after the chicks are hatched. In chicks of equal table quality, those whose body weights increase most rapidly during the first 8 to 12 weeks of life are generally considered the most profitable. More food is required for maintenance in the slower growing birds and turn-over of investment is not so rapid when growth is slow. These are probably some of the reasons why periodic increase in body weight is used in so large a number of studies as the measure of economic efficiency in the growth of chickens. It is evident that the most rapidly growing chickens do not, by virtue of their growth rate, necessarily have a body form that is pleasing to the eye.

## METHOD OF SECURING DATA

Many investigators have demonstrated that season of hatch influences the growth rate of chickens. Kempster and Parker (11) have explained the differences in growth rate of their chicks as the result of retardation produced by high summer temperatures. Galpin (6), raising chickens under controlled temperatures, has suggested that the main factors influencing growth rate are vested in the egg, and these in turn, are probably related to factors controlling the physiological activity of the dam. Since seasonal differences might affect proportions as well as general body growth, the records used for this report were obtained for different kinds of chickens hatched at the same time and subjected to the same environment.

In order that the study might be applicable to the production of broiler and frying chickens in Oklahoma, 1,024 chicks were hatched

<sup>1</sup> Received for publication April 2, 1940.

<sup>2</sup> Italic numbers in parentheses refer to Literature Cited, p. 442.

on November 10, 1938. Classified according to parentage, these chicks belonged to five groups, produced by the following sires and dams:

<i>Sire</i>	<i>Dam</i>
Group 1, White Plymouth Rock	× White Plymouth Rock.
Group 2, Single-Comb White Leghorn	× Single-Comb White Leghorn.
Group 3, Dark Cornish	× White Plymouth Rock.
Group 4, Dark Cornish	× Single-Comb White Leghorn.
Group 5, White Plymouth Rock	× Single-Comb White Leghorn.

Hereafter reference to the different progeny groups is made by the breed name of the parents, as Plymouth Rock, Leghorn, Cornish × Plymouth Rock, Cornish × Leghorn, and Plymouth Rock × Leghorn.

To make the crossbreds directly comparable with the Standard varieties, the following procedure was used. An equal number of Cornish and Plymouth Rock males were alternated daily between two pens of Plymouth Rock females. These pens produced groups (1) and (3) given above. The crossbreds were distinguished by colored plumage and pea comb. Groups 4 and 5 were produced by daily alternating the respective males between two pens of Leghorn females. The crossbred chicks in this case were identified by the pea comb. This identification was checked at 8 weeks of age by the dominant, compact or close feathering characteristic of crosses involving the Cornish breed. The Leghorn chicks (group 2) were produced by alternating a number of males in pens of females whose breeding was similar to that of the females used for the production of groups 4 and 5. The Leghorn breed is not ordinarily considered desirable for the production of poultry meat, but because of a shortage of eggs from larger breeds in September and October, many commercial producers of chicks have been seeking information regarding the advisability of crossing large males with White Leghorn females. It was for this reason that the particular crosses to be included in these observations were chosen. The White Leghorn and White Plymouth Rock stock conform very closely to the breed standards as given by the American Poultry Association (2). The Dark Cornish used in these studies deviate from the Standard type by being somewhat longer legged and approaching the body carriage of the Game or Malay fowl.

To control environmental differences a proportionate number of each of the 5 groups was placed in each of 12 pens in a continuous floor brooding system. Thus each group as a whole was subjected to the same environment. For the first 4 weeks the chicks were fed an all-mash ration, No. CQ263.<sup>3</sup> At 4 weeks of age an additional feeder containing 2 parts cracked yellow corn, 1 part wheat, and 1 part kafir was placed in each pen. When the chickens reached 12 weeks of age, sex was recorded. At this time 400 of the living birds were retained for further observation and the remainder killed and plucked for visual observations on body form.

Individual body weights were recorded at hatching and every 2 weeks during the experiment. Shank length, anterior body depth, and keel length were measured at 8, 12, 16, and 20 weeks after hatching. Since previous unpublished records indicated that the female shank ceases increasing in length by about 4 months of age, shanks of

<sup>3</sup> PENQUITE, ROBERT, HELLER, V. G., and THOMPSON, R. B. RATIONS FOR THE PRODUCTION OF MEAT BIRDS, BROILERS, FRYERS AND BOOSTERS. Okla. Agr. Expt. Sta. Min. Cir. 32, [4] pp., illus. 1939.

all females retained for study were measured every 2 weeks between 16 and 24 weeks of age.

#### METHODS OF SUMMARIZING DATA

The shape ratio

$$\frac{\text{Linear measurement}}{\sqrt[3]{\text{body weight}}} \quad (1)$$

is used to obtain a numerical expression of body form. While under certain conditions this ratio may be shown mathematically to be a function of the allometric or relative growth equation (19), it is used in this paper only as a simplified index of conformation. Lerner (18, 19) has criticized this ratio on the basis that it is in reality the initial growth index of Huxley and Teissier (5) and that it is a selected case of limiting allometry. Formula 1 presents no evidence as to how the shape of the chicken was attained. Since it has been shown that shank length exhibits positive allometry as compared with the body as a whole (16), the numerical value of the shape ratio when based on shank length would be expected to have different numerical values with increases in age of the bird. The value of the shape ratio as a means of predicting conformation differences was checked against a visual classification on those chickens that were killed and plucked at 12 weeks of age.

Shape ratios used in a previous report (8) were calculated for each linear measurement of each female. In the present data the shape ratios were obtained from means. As a test of validity of this procedure data for the Plymouth Rock females included in this report were calculated by both methods. The slight difference between the mean of ratios and the ratio of the means proved to be statistically nonsignificant. Analyses of variance were made from the original measurements. From the statistics thus obtained and the mean body weights of the groups being compared probability points were estimated.

To measure the relative growth of the part as compared with the body as a whole, a growth ratio was obtained from the equation

$$\frac{\ln L_2 - \ln L_1}{\ln W_2 - \ln W_1} \quad (2)$$

in which  $W_1$  and  $L_1$  are mean body weight and mean linear measurement, respectively, at the beginning, and  $W_2$  and  $L_2$  mean weight and mean measurement of the same group at the end of the growth period under consideration. Since these growth ratios are based on mean measurements from relatively large numbers of individuals, they probably present a fairly accurate estimate of relative growth in each group during the period of consideration.

Formula 2 represents one method of estimating the value of  $\alpha$  in the allometric equation (5),  $y = bx^\alpha$ , when  $y$  is a part,  $x$  the standard or whole, and  $b$  the value of  $y$  when  $x=1$ . Many investigators have stressed that more nearly precise approximations of a constant growth ratio ( $\alpha$ ) are obtained from least-squares solutions which are based upon a number of points during growth. Solutions of formula 2 must not be confused with actual equilibrium constants for early



phases of growth. They are used simply to portray the relative growth of the part during the period under consideration and for comparison with changes in the shape ratio (formula 1) calculated at the beginning and end of the period. An expedient tool in practice is one which permits calculation at a given point in time.

### SUMMARIZED DATA

#### CESSATION OF GROWTH IN LENGTH OF SHANK

Table 1 contains the mean shank length of 8 groups of females at 2-week intervals between 16 and 24 weeks after hatching. It may be observed that the shank in each group, except the Light Brahmas, reached its maximum length by 16 to 18 weeks. Shank length of the Light Brahmas increased up to 22 weeks of age. In all other groups the small numerical differences in shank length at successive periods after 18 weeks are well within the error of measurement. In view of the observation of Waters (27) it is probable that the apparent retarded growth of shank in the Light Brahma may be the result of a general retardation associated with late sexual maturity. Since there is very little information in the literature concerning the period of longitudinal shank growth further observations are in progress for both sexes.

The records presented in table 1 are in agreement with those of Latimer (15) for the tarsometatarsal length in White Leghorns. Latimer observed that the tarsometatarsus ceases to grow in length by 110 days after hatching in females and 142 days in males. Bone weight, however, increases to maturity. These findings would tend to invalidate the assumption of Lerner (16) that length ( $L$ ) and weight ( $W$ ) of the bird's tarsometatarsus bear a consistent relation as expressed by  $W = CL^3$ .

TABLE 1.—Mean shank length of growing female chickens of different breeds and crosses aged 16 to 24 weeks

Breed or cross of parents	Females	Shank length for indicated age (weeks)				
		16	18	20	22	24
	Number	Centi- meters	Centi- meters	Centi- meters	Centi- meters	Centi- meters
Single-Comb Brown Leghorn	28	8.86	8.89	8.94	8.89	8.89
Rhode Island Red	14	10.36	10.57	10.64	10.59	10.59
Light Brahma	5	10.03	10.39	10.57	10.77	10.77
White Plymouth Rock	44	10.52	10.69	10.69	10.69	10.67
Dark Cornish male×White Plymouth Rock female	46	9.98	10.26	10.31	10.34	10.34
Single-Comb White Leghorn	37	9.47	9.63	9.55	9.60	9.58
Dark Cornish male×Single-Comb White Leghorn female	17	9.32	9.50	9.47	9.47	9.47
White Plymouth Rock male×Single-Comb White Leghorn female	36	10.06	10.19	10.16	10.13	10.19

Records on the increase in shank length with age of White Leghorns collected by Neunteufel (25) indicate that growth is very slow after 22 weeks. In Neunteufel's data the number of birds varied slightly with successive measurements, which may account for the discrepancy between her records and those presented in this paper. Lerner's data (16, 17, 18) indicate that shank length of both males and females

increased more rapidly than body weight in chickens between 16 and 20 weeks of age. The possibility of an earlier attainment of maximum shank length in more rapidly growing stock should be tested by further research.

Since the shank appears to reach its maximum length relatively early, normal increases in general body weight after this period will produce marked changes in a shank-body shape ratio (formula 1). The cessation of shank growth at an early age is not a phenomenon peculiar to chickens. Unpublished records of mean shank length of female turkeys show no increase between 24 and 52 weeks after hatching. Pålsson (26) observed that the cannon (carpal) bone of sheep reached its ultimate length by approximately 1 year but was still actively growing in thickness. Use of shank length in a shape ratio to determine whether hereditary conformation differences exist must, therefore, be at known ages.

Since Lumer (20) has demonstrated that the allometric equation is not valid when either of the variables approaches its upper asymptote, growth constants (5) for the relation between shank and body apply only during early phases of growth, at least prior to 16 weeks in females. The limiting equilibrium constant for adult proportions differs from the growth constant as a result of the normal growth of body after increases in shank length cease. Should the shank-body growth constant of Bantams be similar to that of normal sized breeds, a much lower limiting equilibrium constant is indicated from the data of Lerner (16).

Two factors which restrict length of the long bones to a greater degree than body weight have been studied by Landauer (12, 13, 14). From previous hatchability records the rather long-legged Cornish used in the present studies appeared not to carry the lethal factor (13) responsible for shortening of the long bones. Wright (28) has suggested special factors which control the growth of parts. Lumer (21) observed six allometric tribes in domestic dogs. Special factors such as these occurring in some strains and not in others may tend to depreciate the value of shank length in an expression of body form. In other words, anything which restricts the length of the long bones as compared with the remainder of the skeleton in certain strains would invalidate the use of shank length for comparing skeletal size of all strains of chickens.

#### GENERAL BODY GROWTH

The chickens used in this study were started on a diet<sup>4</sup> that has produced rapid early growth (table 2). Moreover, they were hatched at a season of the year that appears to be very favorable for rapid early increases in weight. As a result, growth rate of the body as a whole was greater than that ordinarily reported. The percentage growth rates calculated according to the arithmetical method of Brody (3) are presented in table 2. In males, percentage growth rate from 0-4 weeks of age ranges from 148.3 for the Cornish  $\times$  Plymouth Rock cross to 153.4 for the Cornish  $\times$  Leghorn cross. The pullet growth rates during the same period ranged from 143.9 for the Cornish  $\times$  Plymouth Rock progeny to 150.0 percent for progeny of the Plymouth Rock  $\times$  Leghorn cross. These growth rate figures are considerably higher than those given by Kempster and Parker (11) for

<sup>4</sup> See footnote 3.

early hatched pullets. Similarly, the growth rate of both sexes from 4 to 8 and 8 to 12 weeks may be considered above normal. When the logarithms of body weights at 2-week intervals are plotted against units of time, these data do not approach a straight line relation at any time during the period of observation to 18 weeks. Therefore, body weight in this study did not increase at a constant percentage rate.

TABLE 2.—Percentage growth rates<sup>1</sup> of chickens used in this study while on the starting diet

Breed or cross	Males	Growth rate for indicated age period (weeks)			Females	Growth rate for indicated age period (weeks)		
		0-4	4-8	8-12		0-4	4-8	8-12
Dark Cornish × White Plymouth Rock	Number 89	Percent 148.3	Percent 99.5	Percent 58.8	Number 105	Percent 143.9	Percent 95.9	Percent 54.6
White Plymouth Rock	164	150.2	101.1	58.9	151	148.2	95.8	54.0
White Plymouth Rock × Single-Comb White Leghorn	134	153.3	101.1	56.9	126	150.0	94.6	50.1
Dark Cornish × Single-Comb White Leghorn	25	153.4	100.3	52.9	44	147.9	93.6	50.7
Single-Comb White Leghorn	63	149.3	94.0	54.8	64	147.3	87.4	45.6

<sup>1</sup> Percentage rate of growth =  $\frac{(W_2 - W_1)}{\frac{1}{2}(W_1 + W_2)} \times 100$ , when  $W_1$  is body weight at the beginning and  $W_2$  weight at end of the period under consideration.

In each of the five groups, the male chicks weighed more than the females at 2 weeks of age. This mean difference between sexes within groups was highly significant statistically. Whether this was due to an early difference in the sex stimulus for growth remains to be determined. Although Jourdain (9) obtained more rapid growth in pullets raised separately from the cockerels, it is inconceivable to the author that the presence of male chicks in these groups could have influenced the growth rate of their sisters at such an early age. Munro and Kosin (24) have observed a sex difference in chick weight at hatching. Ackerson and Mussehl (1) found males to be heavier than females at 1 week of age. Under restricted diet and an integrated value for growth based on two lots hatched 1 day apart, Mstislavsky (23)<sup>5</sup> observed slightly superior growth of males during the first 15 days after hatching. This difference then disappeared and was not again observed until approximately the forty-fifth day. Studies are in progress to determine whether a ration which produces maximum early growth favors an early expression of the sex difference in growth rate when sexes are reared separately from hatching.

Mortality to 12 weeks of age was relatively low. In each of the Plymouth Rock and Plymouth Rock × Leghorn groups the loss was 6.5 percent. Mortality in Leghorns was 3.8 percent; in Cornish × Leghorn, 1.5 percent; and in Cornish × Plymouth Rock, 5.8 percent. Data for chicks that died are excluded from the summarized records.

#### RELATIVE GROWTH OF SHANK AND BODY

Table 3 presents the available material relative to changes in body shape which result from the relative rate of shank growth. Assuming

<sup>5</sup> The author is indebted to Dr. V. L. Maleev of the Engineering Division, Oklahoma Agricultural and Mechanical College, for interpretation of this part of the cited article.

that a growth ratio of 0.333 (16) represents equal growth rate of shank and body between 8 and 12 weeks after hatching, shank length increased more rapidly than body weight in all cases except Cornish  $\times$  Leghorn and Cornish  $\times$  Plymouth Rock females. By the method described previously it is estimated that the positive allometry of shank length observed in Leghorn females and all males between 8 and 12 weeks is statistically significant.

Lerner (18) has criticized the author's use of the shape ratio, stating that it is less precise than solutions based on logarithms. A very consistent relation may be noted between the value of the growth ratio (formula 2) and the increase in numerical value of the shape ratio (formula 1) for the 8- to 12-week period. The data shown in tables 3 and 4 clearly indicate that the differences in the shape ratios that are calculated arithmetically are as sensitive a measure of growth changes as the growth ratio calculated from the logarithms of the data at two points in the growth period. It is possible that this close association may be observed only when birds of the same age are being compared.

TABLE 3.—*Relative growth of shank length and body weight with resulting changes in body shape of chickens between 8 and 16 weeks of age*

Sex, breed, or cross	Shape ratio <sup>1</sup> at 8 weeks	Growth ratio <sup>2</sup> at 8-12 weeks	Shape ratio <sup>1</sup> at 12 weeks	Growth ratio <sup>2</sup> at 12-16 weeks	Shape ratio <sup>1</sup> at 16 weeks	Birds of superior shape at 12 weeks
Females:						Percent
Dark Cornish $\times$ White Plymouth Rock	587	0.336	588	0.265	575	79
White Plymouth Rock	591	.346	595	.265	585	71
Dark Cornish $\times$ Single-Comb White Leghorn	584	.334	585	.169	555	91
White Plymouth Rock $\times$ Single-Comb White Leghorn	598	.341	601	.248	585	58
Single-Comb White Leghorn	604	.372	615	.207	590	30
Males:						
Dark Cornish $\times$ White Plymouth Rock	599	.375	616	.341	618	61
White Plymouth Rock	613	.369	626	.370	636	48
Dark Cornish $\times$ Single-Comb White Leghorn	600	.366	611	.330	610	-----
White Plymouth Rock $\times$ Single-Comb White Leghorn	618	.359	628	.326	626	58
Single-Comb White Leghorn	624	.348	629	.339	630	42

<sup>1</sup> By formula 1, p. 433.

<sup>2</sup> By formula 2, p. 433.

The data presented here tend to support Lerner's assumption (16) that a value of 0.333 for the growth ratio represents relatively equal increases in shank length and body weight, i. e. isometry. In table 3 the column labeled "Birds of superior shape at 12 weeks" gives the percentage of those killed at 12 weeks of age that appeared superior in body conformation after feathers had been removed. A fair agreement between the shank-body shape ratio at 12 weeks and the visual grading may be observed within each sex. A decrease in the numerical value of the shape ratio is associated with a higher percentage attaining a superior rating on visual body shape.

Since the shank-growth ratio for females between 12 and 16 weeks of age is smaller than 0.333 it is apparent that the body grew more rapidly than the shank increased in length. The resulting changes in body form may be seen in the numerical reduction of the shank-body shape ratio at 16 weeks of age in females. During this same period growth rate of the male shank equaled or exceeded body growth;

however, a marked reduction may be observed in the relative increase in shank length between 12 and 16 weeks of all male groups, except the Plymouth Rock. Since these males were used to obtain other information complete records are not available at 20 weeks of age. The body weight of those males measured at 20 weeks had increased more rapidly than shank length. The data presented in table 3 show in a more marked degree similar fluctuations in relative growth values observed by Lerner (17) for individual birds. They do not invalidate the use of a constant growth ratio for early stages of growth but they do indicate that approximately 12 weeks of age may be the upper age limit for its use with females.

#### BODY FORM AS INFLUENCED BY RELATIVE CHANGES IN DEPTH

Body depth is here considered as the minimum distance between the anterior extremity of the keel and the dorsal surface of the body. The depth-body shape ratios and growth ratios calculated from formulas 1 and 2 are given in table 4. Between 8 and 12 weeks body depth increased relatively much more slowly than body weight. As a result the depth-body shape ratios are much smaller at 12 weeks. The body of all varieties and both sexes, therefore, became much plumper by 12 than they were at 8 weeks after hatching. After this period body depth and weight appeared to increase in about the same percentage relation. The majority of the differences between shape ratios at 12 and 16 weeks may be considered statistically nonsignificant.

TABLE 4.—*Relative growth of body depth and weight of chickens, and resulting changes in body shape*

Sex, breed, or cross	Shape ratio <sup>1</sup> at 8 weeks	Growth ratio <sup>2</sup> at 8-12 weeks	Shape ratio <sup>1</sup> at 12 weeks	Growth ratio <sup>2</sup> at 12-16 weeks	Shape ratio <sup>1</sup> at 16 weeks	Growth ratio <sup>2</sup> at 16-20 weeks	Shape ratio <sup>1</sup> at 20 weeks
<b>Females:</b>							
Dark Cornish × White Plymouth Rock	575	0.252	550	0.364	556	0.298	551
White Plymouth Rock	590	.266	568	.376	577	.348	579
Dark Cornish × Single-Comb White Leghorn	580	.226	549	.323	548	.247	536
White Plymouth Rock × Single-Comb White Leghorn	592	.225	580	.317	557	.332	557
Single-Comb White Leghorn	598	.157	552	.326	550	.308	547
<b>Males:</b>							
Dark Cornish × White Plymouth Rock	578	.272	558	.368	568		
White Plymouth Rock	586	.291	571	.356	577		
Dark Cornish × Single-Comb White Leghorn	578	.258	555	.345	558		
White Plymouth Rock × Single-Comb White Leghorn	593	.260	568	.341	569		
Single-Comb White Leghorn	597	.205	555	.324	553		

<sup>1</sup> By Formula 1, p. 433.

<sup>2</sup> By Formula 2, p. 433.

#### CHANGES IN RELATIVE KEEL LENGTH

It has previously been shown (7) that changes in relative keel length do not materially affect the visual conformation of the body. Between 8 and 12 weeks of age there was very little change in proportional keel length, but between 12 and 16 weeks keels of both sexes in all groups became proportionally much longer. During this period the mean shape ratios of the females increased from 509 to

570 and the males from 551 to 571. These data indicate that the growth of the keel differed distinctly from that of shank length. Further evidence of an inherent difference between growth in length of keel and growth in length of the long bones is found in the data of Caskey and Norris (4). In these experiments a deficiency of manganese during the embryonic stages limited the postnatal growth of the long bones but did not influence the length of the keel. The close agreement between the differences in shape ratios at the beginning and end of each period and the growth ratios for that period was also observed in the data for keel length. Isometry, or relatively equal growth of the part to the whole in all three linear measurements (shank length, body depth, and keel length), appears to give a growth ratio of approximately 0.333.

#### HERITABLE SHAPE DIFFERENCES BETWEEN BREEDS AND CROSSES

It has been impossible by the use of weight, depth, shank and keel length to detect significant differences in body conformation at 8 weeks of age. Proportional differences in body dimensions are not very large at this age and variability in general is greater. By 12 weeks of age many of the differences in body shape are highly significant as estimated by probability points secured from the actual data. Differences between certain female groups exist in all three criteria. Male groups differed most in proportional body depth at 12 weeks. In both sexes, the Cornish male parent increased the relative body weight as compared with body depth and shank length. The relation between relative keel lengths of the various groups varied through the observation period. The only consistent group difference was the Cornish  $\times$  Leghorn female progeny which had a relatively shorter keel length at 8, 12, 16, and 20 weeks of age.

Proportionally shallow bodies and short shanks indicate superior body shape of chickens after the feathers are removed. On the basis of these two criteria (tables 3 and 4) the visual body shape after killing and dressing would be in the following decreasing order of preference at 12 weeks of age. Males: (1) Cornish  $\times$  Leghorn, (2) Cornish  $\times$  Plymouth Rock, (3) Plymouth Rock  $\times$  Leghorn and Plymouth Rock, and (4) Leghorn; females: (1) Cornish  $\times$  Leghorn, (2) Cornish  $\times$  Plymouth Rock, (3) Plymouth Rock, (4) Plymouth Rock  $\times$  Leghorn, and (5) Leghorn. To test this relation further a random sample of each sex group, except Cornish  $\times$  Leghorn males, was taken and the birds were graded into visual preference groups after they were killed and the feathers removed. The distribution of the birds into 4 arbitrary preference groups is shown in table 5. The plumpest birds were placed in conformation grade 1. Grades 2, 3, and 4 each had inferior shape quality. The guide used to determine shape quality was that given by the Bureau of Agricultural Economics<sup>6</sup>. The percent of the total of each sex group placed in grades 1 and 2 compares very favorably with the order of preference judged from live bird measurements. The relation is not as close in this population at 12 weeks of age as was found for mature cockerels and capons (7).

<sup>6</sup> U. S. DEPARTMENT OF AGRICULTURE, BUREAU OF AGRICULTURAL ECONOMICS. CLASSIFICATION AND TENTATIVE SPECIFICATIONS FOR U. S. STANDARDS AND GRADES FOR DRESSED CHICKEN. 8 pp. Washington, D. C. 1938. [Mimeographed.]

TABLE 5.—*Distribution of birds into 4 arbitrary preference grades according to the number in visual conformation grades after killing and plucking at 12 weeks, and also the percentage of birds in the upper 2 grades*

Breed or cross	Sex	Birds in conformation grade No.				Birds in grades Nos. 1 and 2
		1	2	3	4	
		Num-ber	Num-ber	Num-ber	Num-ber	Per-cent
Dark Cornish × White Plymouth Rock	Male	10	12	10	4	61
	Female	21	21	7	4	79
White Plymouth Rock	Male	14	40	40	19	48
	Female	31	36	19	8	71
White Plymouth Rock × Single-Comb White Leghorn	Male	12	34	23	10	58
	Female	17	29	27	7	58
Dark Cornish × Single-Comb White Leghorn	Male	12	9	1	1	91
	Female	1	9	8	6	42
Single-Comb White Leghorn	Male	1	6	14	2	30
	Female	1	6	14	2	30

From the analyses presented it may be observed that the criteria of body shape which have been used in this study roughly segregate the visual classes. A much greater refinement of measurements and technique is needed to obtain nonsubjective accurate records of body shape in chickens during their early growth period.

#### PERCENTAGE OF EDIBLE FLESH AND BODY CONFORMATION

To determine whether there is any relation between criteria of body conformation and percent of edible flesh, a number of males were deboned and examined. Plymouth Rock and Cornish × Plymouth Rock crossbred males were killed weekly as they reached approximately 6 pounds of live body weight. Another group consisting of Cornish × Leghorn and Plymouth Rock × Leghorn crossbred males were killed as they approached 5 pounds. Edible flesh is considered to include the boneless flesh and skin and the cleaned giblets. The mean shape ratios and mean percentages of edible flesh of dressed body weight are given in table 6. The Cornish parent has caused a statistically significant increase in edible flesh in both crosses. This is in agreement with the observation of Maw (22) for males at 26 weeks of age. The males of the present report ranged between 18 and 27 weeks of age at the time the data were collected. The differences in mean shank ratios correspond very closely to the differences in percentage of flesh of the groups. These differences are highly significant. Within each bird percentage of edible flesh and proportional shank length are not correlated. This may be attributable to a relatively high variability within groups and indicates that the shank-body shape ratio is not a satisfactory basis for predicting the percentage of flesh in individual birds. Jull, Quinn, and Godfrey (10) state that flesh production is apparently inherited largely independently of skeletal structure but present no evidence to support this statement in the case of the chicken.

Percentage of edible flesh is no satisfactory criterion of visual market quality. It appears that a chicken may attain superior body shape in two ways, namely, by an increase in flesh and fat and by changes in the basic shape of the skeleton. Thus far it has been impossible to differentiate birds which attain a plump body through additional flesh and those whose skeleton is so constructed as to make

the body appear plump. It is indicated that the Cornish as a breed has both characteristics and apparently transmits them to its progeny. Actually the difference in percentage of edible flesh in these widely divergent groups ranges from only 1.5 to 1.9 percent of the dressed body weight. Stated in another manner, this mean difference in percent of edible flesh in 5-pound roasters may be expected to be less than one-tenth of a pound. Though this difference is highly significant statistically its value to the consumer is probably less than the value of a desirable body conformation. From the market standpoint it is important that the carcass appear plump regardless of ratio between bone and edible flesh.

TABLE 6.—Mean shape ratios and mean percentage of edible flesh for male birds of different progeny groups when killed at approximately 5 or 6 pounds live weight

Progeny group	Males	Mean dressed weight	Mean live-weight ratios			Ratio of edible flesh to dressed weight
			Depth	Shank	Keel	
	<i>Number</i>	<i>Pounds</i>				<i>Percent</i>
White Plymouth Rock	36	5.37	577	629	606	51.4
Dark Cornish × White Plymouth Rock	41	5.48	576	615	601	52.9
White Plymouth Rock × Single-Comb White Leghorn	20	4.34	586	632	614	51.4
Dark Cornish × Single-Comb White Leghorn	21	4.35	566	601	595	53.3

#### SUMMARY

A thousand and twenty-four chicks were hatched from two breeds and three crosses. These five groups were reared together in such a manner that an equal proportion of each group was subjected to such environmental variations as occur in a uniform brooding system. General body growth was considerably above normal. Male chicks were heavier than their sisters at 2 weeks after hatching.

Longitudinal increases in shank length were observed to cease by 16 to 18 weeks after hatching in all female groups except the Light Brahmas. From these observations it is apparent that the growth constant should be differentiated from that of the limiting equilibrium constant based on relative shank length at maturity. Since shank length rapidly approaches its upper asymptote in growth it is suggested that relative growth constants for the shank length of female chickens may apply only to growth prior to 12 weeks of age. Evidence that 16 weeks may be a comparable age in males is presented.

In this experiment anterior body depth became relatively smaller between 8 and 12 weeks after hatching in both sexes. Extreme differences between relative longitudinal growth of keel and shank were observed.

Heritable difference in conformation manifested between groups could not be readily distinguished until the birds were 12 weeks of age, a fact which demonstrates that a much greater refinement in methods of describing form is needed for growing chicks. The Dark Cornish male parents increased the percent of edible flesh in their crossbred male progeny. Since this increase amounted to only about 1.5 to 1.9 percent of the dressed body weight, body shape is considered more important than edible flesh in poultry practice. Percentage of edible flesh is not satisfactory as a criterion of visual conformation.



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## ANALYSIS OF YIELD OF HARD RED SPRING WHEAT GROWN FROM SEED OF DIFFERENT WEIGHTS AND ORIGIN<sup>1</sup>

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### INTRODUCTION

This paper presents data obtained on plantings of wheat of different kernel size. Two experiments were carried out, the first in 1936, when weather conditions were very adverse. Rates of seeding in this experiment varied from light to very heavy with some of them similar to those used in farm practice. In the second experiment, in 1938, large seed that had matured in the greenhouse and smaller seed that had matured out of doors were space planted.

### REVIEW OF LITERATURE

Many papers have been written dealing with the effect of weight or size of seed upon the resulting crop. As far as the cereals are concerned, most investigators report positive relationship between the crops obtained and the use of heavy and light and large and small parent seeds. Mallach<sup>2</sup> reviewed the literature rather completely and found this to be generally true. In his own experiment with spring wheat and barley the seeds were sorted as to size and then classified according to weight. They were spaced in rows at the rate of about 9 per square foot. Generally the heavier seeds gave the greater grain yield per plant. In 52 comparisons with wheat, the grain yield per plant from the heaviest kernels was 16 percent greater than from the mediumweight kernels. The yield of grain per plant was essentially the same for lightweight and mediumweight kernels. The yield per plant reported by Mallach from the mediumweight and heavyweight seeds are equivalent, respectively, to 60.5 and 70.2 bushels per acre.

Kieselbach and Helm<sup>3</sup> worked with spring and winter wheat and with Kherson oats. Spring wheat, seeded in equal number of kernels per unit area, gave 11 percent greater yield per acre from heavy kernels. When equal weights of seed were planted per row, the difference in yield was scarcely appreciable. The yields were generally between 10 and 12 bushels per acre.

<sup>1</sup> Received for publication June 3, 1940.

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# WHEAT GROWN FROM LIGHTWEIGHT AND FROM HEAVYWEIGHT SEED

## MATERIAL AND METHODS USED

In the spring of 1936 a quantity of wheat which showed marked variation in size of seed was available from the cross Ceres × (Hope × Florence) (breeding number 9.81.45.12). By the use of sieves and by hand picking, two classes of seed grain were prepared, one averaging 26.6 mg. and the other 40 mg. per kernel. The experiment was planned to allow comparisons between rates of seeding differing (1) in number of kernels, with equal seed weights per unit area, and (2) in weight of kernels, with equal numbers of seeds per unit area. The lowest rate of seeding was 11.1 pounds per acre of lightweight seed. As all plantings were made in 8-foot rows, this rate was equal to 0.93 gm. per row of 35 kernels. The next rate was about 50 percent higher, or 54 kernels weighing 1.44 gm. per row, and was essentially the same as the lowest rate for wheat weighing 40. mg. per kernel. The rate of seeding of both classes of wheat was stepped up 50 percent for each increment until 10 rates of seeding were provided for each class. An exception was made between the rates of 406 and 610 kernels per row by the introduction of a rate of 508 kernels, a 25-percent increase in the rate of seeding. See table 1.

TABLE 1.—Weight of seed grain planted when heavyweight and lightweight kernels were used, mature heads developed per kernel planted, and yields per acre secured when equal numbers of kernels were planted per unit area, using 2 classes of seed grain weighing 26.6 mg. and 40 mg. per kernel.

[Differences are shown in weight of grain seeded per acre, the number of mature heads per kernel planted, and the yield in bushels per acre]

Item	Data when indicated number of kernels were planted per row										Average
	35	54	80	120	180	270	406	508	610	916	
Lightweight seed planted per acre.....pounds...	11.1	17.2	25.5	38.3	57.5	86.2	130	162	195	292	
Heads per kernel.....number...	3.11	2.71	2.17	1.69	1.26	.98	.86	.84	.82	.75	1.51
Yield, per acre.....bushels...	9.88	12.95	15.55	14.65	15.13	16.25	15.43	15.85	15.25	11.44	14.24
Heavyweight seed planted per acre.....pounds...	17.2	25.5	38.3	57.5	86.2	130	195	244	292	439	
Heads per kernel.....number...	3.33	2.80	2.23	1.84	1.26	1.02	.94	.88	.83	.76	1.59
Yield, per acre.....bushels...	12.13	14.68	16.08	17.28	17.83	17.90	17.65	18.70	15.28	12.45	16.00
Difference:											
In weight of grain seeded.....pounds...	6.1	8.3	12.8	19.2	28.7	44.0	65.0	82.0	97.0	147.0	
In heads per kernel planted.....number...	.22	.09	.06	.25	0	.04	.08	.04	.01	.01	.08
In yield per acre.....bushels...	2.25	1.73	.53	2.63	2.70	1.65	2.22	2.85	0.03	1.01	1.76

Provision was thus made for 10 comparisons of results obtained from equal numbers of kernels per row and for 8 comparisons from equal weights of seed grain per row. Each planting was repeated 4 times, thus making 80 rows in the experiment proper, and these were randomized in 4 blocks. Each yield row was provided with a pair of guard rows seeded with a drill at the usual rate of 75 pounds per acre. A stand count taken 25 days after seeding showed that 98 percent of the seed had developed plants with essentially no difference in average stand from the 2 weights of seed or from the different rates of seeding.

## SEASONAL CONDITIONS

Weather conditions during the growing season as a whole, and especially during July, were by far the worst on record. The excess daily temperature above normal from May through July averaged 7° F., while the daily excess for July was nearly 12°. The rainfall for the 3 months was 21 percent of normal, and for July it was at the record low of 13 percent of normal.

## EXPERIMENTAL RESULTS

Table 1 shows the rates of planting, the heads produced per kernel planted, and the yields for the 10 comparisons. Three sets of differences are also shown. Kernels planted per row were the same for the 2 weights of seed.

In all cases the heavy kernels gave yields larger than the light kernels when the results obtained from seeding equal numbers are compared. The average difference in yield of 1.76 bushels is very significant indeed. It is not evident that the continually greater quantities of seed used per acre had any corresponding effect on differences in yield. In all cases the weight of the heavy seed planted was approximately 50 percent greater than that of the light seed. The heads produced per kernel planted are slightly greater for the heavy-kernel seeding; and, while the average difference is but 0.08 head per kernel, this difference is significant with odds of 60 to 1. The significance of these differences is calculated from a total of 79° of freedom with 57 for error.

It is quite evident from table 1 that yields maintain themselves with various rates of seeding to about 200 pounds per acre. Indeed, with the 40-mg. seed the maximum yield resulted from 244 pounds of seed per acre, which is about three and one-fourth times the quantity usually used in farm practice. One might have presumed that such heavy seeding under the adverse weather conditions that obtained would be inimical to good yields, in comparison with lower rates; but it evidently was not.

The comparison of the results obtained from equal weights of seed grain per acre, the number of kernels differing for each weight class, is shown in table 2.

TABLE 2.—Number of heavyweight and of lightweight kernels planted, mature heads developed per kernel planted, and yields per acre secured when equal weights of seed grain weighing 26.6 mg. and 40 mg. per kernel are planted per unit area

[Differences are shown of weight of grain seeded per acre, the number of mature heads per kernel planted and the yield in bushels per acre]

Item	Data when indicated quantity of seed was planted per acre								Average
	17.2 pounds	25.5 pounds	38.3 pounds	57.5 pounds	86.2 pounds	130 pounds	195 pounds	292 pounds	
Kernels of lightweight seed planted per row.....number	54	80	120	180	270	406	610	916	
Heads per kernel.....do.	2.71	2.17	1.59	1.26	0.98	0.86	0.82	0.75	1.39
Yield, per acre.....bushels	12.95	15.55	14.65	15.13	16.25	15.43	15.25	11.43	14.58
Kernels of heavyweight seed planted per row.....number	35	54	80	120	180	270	406	610	
Heads per kernel.....do.	3.33	2.80	2.23	1.84	1.26	1.02	0.94	0.83	1.78
Yield, per acre.....bushels	12.13	14.68	16.08	17.28	17.83	17.90	17.65	15.28	16.10
Difference:									
In kernels planted per row.....number	-19	-26	-40	-60	-90	-136	-204	-306	
In heads per kernel planted.....do.	0.62	0.63	0.64	0.58	0.28	0.16	0.11	0.08	0.39
In yield, per acre bushels	-0.82	-0.87	1.43	2.15	1.58	2.47	2.40	3.85	1.52

On this basis only eight comparisons are possible. Because equal weights of light and heavy seed were planted, some of the rows of lightweight seed planted at the lower rates may have had an advantage in that more kernels were planted per row. On the other hand, for those rows receiving the heavier seeding the greater number of kernels may have resulted in disadvantage due to overcrowding of plants. That overcrowding actually occurred is indicated by the yield differences. With a seeding rate of 17.2 pounds per acre a heavier yield is obtained from the lightweight kernels, whereas the contrary is true when the rate of seeding is 292 pounds per acre. The net gain in yield with the heavier weight of seeding is  $4.7 \pm 1.2$  <sup>4</sup> bushels per acre. In a similar comparison, when the rates of 25.5 and 195 pounds per acre are considered, the net gain in yield with the heavier weight of seeding is  $3.3 \pm 1.5$  bushels per acre. This greater yield from heavy kernels was in evidence and of significance in the three sets of rows planted at rates comparable with field rates, that is, from 38 to 86 pounds per acre, with an average excess of  $1.7 \pm 0.6$  bushels. In these rows seeded at rates comparable with usual field rates, the number of lightweight kernels planted per row was not excessive, and crowding to the detriment of the seedlings and more mature plants could hardly have occurred more than easily may occur in the field. The number of heads produced per kernel planted from the larger, and more thinly planted, kernels was greater by an average of 0.39 when comparisons are made between results obtained from equal weights of seed planted per row. This difference is very significant.

The size of the kernels harvested is of interest in view of the severe seasonal conditions. The results, comparing the rows in which equal numbers of kernels were planted are shown in table 3.

TABLE 3.—Weight of kernels harvested from 2 sets of plantings from seed of lightweight and of heavyweight kernels, with comparisons between rows planted with equal numbers of kernels

Item	Data where indicated number of kernels were planted per row										Average
	35	54	80	120	180	270	406	508	610	916	
Weight of harvested kernels from—											
26.6-mg. kernels.....	<i>Mg.</i> 23.7	<i>Mg.</i> 24.0	<i>Mg.</i> 25.4	<i>Mg.</i> 24.6	<i>Mg.</i> 24.1	<i>Mg.</i> 25.3	<i>Mg.</i> 23.8	<i>Mg.</i> 24.6	<i>Mg.</i> 24.4	<i>Mg.</i> 21.4	<i>Mg.</i> 24.1
40.0-mg. kernels.....	25.1	24.9	25.8	27.1	27.6	26.2	25.4	24.6	23.7	23.4	25.4
Difference.....	1.4	.9	.4	2.5	3.5	.9	1.6	0	.7	2.0	1.3

The mean difference in kernel weight from the two crops is 1.3 mg. in favor of the heavy-kernelled plants. The analysis of variance indicates that this is distinctly significant. The difference in kernel weight when a similar comparison is made between equal weights of seed grain planted per row is 1.6 mg., which is likewise significant.

Mallach<sup>5</sup> did not find consistent differences in the kernel weight of grain in plants grown from heavyweight and lightweight seed. He cited two workers who had secured similar results. In the work herein reported the crop secured from the seed planted with heavy kernels had very significantly heavier kernels.

<sup>4</sup> Standard errors are based on the analysis of variance.

<sup>5</sup> MALLACH, JOSEF. See footnote 2.

One explanation for the fact that the heavy-kerneled seed resulted in a crop with heavier kernels might be that the seed used was not homozygous with respect to seed weight and that a genetic separation had been made automatically in separating for kernel size preparatory to seeding. The possibility of this having occurred seems rather remote. Another explanation is possible. In 1934 certain sister selections of the wheat used in this experiment were grown in a yield trial, and it was found that kernel weight increased with yield with a regression coefficient of 0.86, which was significant. If the proper regression formula were used in this case, the change in kernel weight corresponding to the above changes in yield would be 1.5 mg. This difference agrees very well with those shown in table 3. It seems likely, then, that the difference in kernel weight of the crop is to be associated with differences in yield.

#### DISCUSSION

The data presented show distinct differences in yield in the wheat crop grown from heavyweight and from lightweight kernels. This result was obtained when the same weights of seed grain and also when the same number of seeds was planted per unit area. In the first instance the yield per acre was 10 percent greater from the seed of the heavy kernels and in the other it was 12 percent greater.

The results secured by Kiesselbach and Helm,<sup>6</sup> already mentioned, are very similar as regards spring wheat to those reported here. Yields of spring wheat reported by them were relatively low and were even less than those secured at Fargo in 1936 when the weather conditions were so very adverse. Their results from the use of large and small kernels of winter wheat were less striking. As an average for 4 years the advantage in yield from the larger kernels was only 5 percent with an average of about 35 bushels per acre. Thus, with better conditions, shown by larger yields, the heavy kernels showed less advantage. Mallach<sup>7</sup> states that favorable environmental conditions tend to eliminate differences in plant growth from seeds of different weight. This seems to have held with spring and winter wheat in Nebraska. In the Fargo experiment, with two lots of seed taken from a presumably pure line, it was possible to obtain the rather extreme kernel weights of 26.6 and 40 mg. This disparity in weight of seed kernels in the experiment herein reported is doubtless much greater than could be obtained on the farm by seed-cleaning machinery with a single lot of grain.

#### WHEAT GROWN FROM SEED MATURING IN THE GREENHOUSE AND IN THE OPEN

In the writer's experience, wheat that has matured in the greenhouse under favorable conditions produces a crop with kernels larger and of finer appearance than wheat that has matured in the open. The question is, how does a crop produced from greenhouse seed differ from one produced from ordinary seed?

<sup>6</sup> KIESELBACH, T. A. and HELM, C. A. See footnote 3.

<sup>7</sup> MALLACH, JOSEF. See footnote 2.



## MATERIAL AND METHODS USED

In the fall of 1937 families of wheat were available from different crosses made in different years. In each of these families a group of hybrid selections was available for experimental use. Seed of a number of these selections within a number of families was planted in the greenhouse. For each group planted in the greenhouse seed of sib selections was held in reserve for planting the following spring. There were 16 hybrid families all told, with 8 hybrid selections of each grown in the greenhouse and seed of 8 sibs held in reserve. In the spring of 1938 16 hybrid selections were thus available for planting from each of the 16 hybrid families. The 2 lots of 8 in each family were presumably of essentially equal genetic value. One lot in each case had been grown an additional generation. The seed which had been held in reserve from 1937 will be designated outside seed. Seed from the plants grown under glass during the winter of 1937-38 will be known as greenhouse seed. Ceres and Thatcher varieties were included as checks, making 18 groups in all. The 16 hybrid families and the 2 varieties will be called families for convenience.

The various hybrid families are found in table 4, with their breeding and their genetic age.

TABLE 4.—*List of wheat hybrids used in an experiment with seed matured out of doors compared with seed matured in the greenhouse*

No.	Hybrid	Origin	Generation
1	3.2.3.30	R.L.625 × CHF 2754	F <sub>6</sub> , F <sub>7</sub>
2	3.2.3.33		
3	3.2.3.64		
4	3.2.3.67		
5	3.2.3.85		
6	5.7	Mercury × (H-44 × Ceres)	F <sub>6</sub> , F <sub>8</sub>
7	6.19.7	Mercury × (Mercury × H-44 Ceres)	F <sub>6</sub> , F <sub>8</sub>
8	6.19.7.29	Mercury × (Mercury × H-44 Ceres) × (625 × 2754)	F <sub>6</sub> , F <sub>8</sub>
9	5.9	Mercury × (Reliance × Hope)	F <sub>6</sub> , F <sub>8</sub>
10	6.19.9	Mercury × (Mercury × Reliance-Hope)	F <sub>6</sub> , F <sub>8</sub>
11	6.19.9.29	Mercury × (Mercury × Reliance-Hope) × (625 × 2754)	F <sub>6</sub> , F <sub>8</sub>
12	6.19.6	Mercury × (Mercury × (Comet × (Hard Federation × Hussar)))	F <sub>6</sub> , F <sub>8</sub>
13	6.19.6.29	Mercury × (Mercury × (Comet × (Hard Federation × Hussar))) × (625 × 2754)	F <sub>6</sub> , F <sub>8</sub>
14	6.19.0	Mercury × (Mercury × (Hope × Marquis))	F <sub>6</sub> , F <sub>8</sub>
15	6.19.0.29	Mercury × (Mercury × (Hope × Marquis) × (625 × 2754))	F <sub>6</sub> , F <sub>8</sub>
16	6.18-11.29	(Reliance × Reward) × (625 × 2754)	F <sub>6</sub> , F <sub>8</sub>

The maturing of larger kernels from wheat grown in the greenhouse under favorable conditions is probably due mainly to cooler temperatures and controlled heat. In half of the greenhouse, where the plants were grown in 6-inch pots, three to the pot, the temperature was allowed to run higher, and as a result the seed was smaller and quite comparable to seed produced in the open. Ceres and Thatcher were grown in the greenhouse to furnish seed for the experiment, but as these plants were also subject to temperatures above the optimum no marked excess in size over the outside seed was in evidence. This was also true for five of the hybrid families grown in pots. As a result, the kernel weight of the greenhouse seed was in a few cases less than that of the comparable outside seed, and in one instance decidedly so. The distribution of excess kernel weight of the seed from plants maturing in the greenhouse over the corresponding outside seed is as follows, expressed as milligrams per kernel.

Excess of seed weight:	Number of pairs
-3	2
-2 to +1	3
2 to 5	2
6 to 9	5
10 to 13	4
+14	2

In the three groups with excess kernel weights centering at 3.5, 7.5, and 11.5 mg. the greenhouse seed showed excess weights of 17, 24, and 36 percent, respectively. Thus in most cases there was a rather pronounced difference in weight between the two classes of seeds.

The seeds were space-planted 2 inches apart in unguarded rows 8 feet long and 12 inches apart. The seed, previously taped in the laboratory by the device invented and constructed by Glenn Smith,<sup>8</sup> was planted in tape form with a Columbia drill, modified by Smith for the purpose. This method greatly facilitated space planting. The 36 planting units each had 8 subunits, as indicated above. The set-up of the experiment was a 6 by 6 square lattice, following the plan of Yates,<sup>9</sup> with 4 sets of *X* and 4 of *Y* blocks. The outside seed of 3.2.3.30, for example, consisted of 8 selection units, 7, 8, 10, 11, 16, 22, 34, and 41, of which 4 were in *X* blocks and 4 in *Y* blocks.

Seedling counts were taken May 10, when the stand was found to be 96.4 percent of the seeds planted. The stand from the greenhouse seed was 0.2 percent greater than that from the outside seed, a difference which lacked significance. Plants from the greenhouse seed headed 0.2 of a day earlier than those from the outside seed, a difference which was just significant.

The plants were pulled at harvesttime, the two end plants of each row discarded, and the remaining plants wrapped in a bundle and laid away until a study could be made. At that time the plants and the fertile culms were counted, and a random sample of 50 heads were taken, omitting any heads obviously defective. Notes were also taken as to disease present. (Rust notes had been taken previous to harvest.) Occasional plants killed by root rot were discarded. In general, rust was of little moment as most of the wheats were resistant. Exceptions were Thatcher, attacked by leaf rust, and Ceres and the hybrid 6.18-11.29, attacked by stem rust. The bundles were threshed in a small machine which allowed all seed to be saved. The 50-head samples were weighed and counts were made of total spikelets, fertile spikelets, sterile spikelets, and three-kerneled spikelets. The wheat from the 50 heads was weighed, the kernel weight was taken, and an estimate of kernels per head was made. The above work was done for each of the 288 bundles.

In calculating bushels per acre the rows were estimated at full stands of 48 plants per 8 feet. The occasional missing plants had little influence upon yield as the correlation coefficient calculated between plants per row and yield was found to be 0.14. Omitting Ceres and Thatcher, the varieties having the fewest plants per row, the coefficient was -0.04. If differences in stand had influenced yield per plant in such a way that fewer plants per unit area had developed larger individual yields then a significant negative correlation coefficient would have been expected.

<sup>8</sup> SMITH, GLENN S. GUMMED-PAPER TAPE FOR SPACE-PLANTING WHEAT. *Amer. Soc. Agron. Jour.* 30: 348-352, illus. 1938.

<sup>9</sup> YATES, F. A NEW METHOD OF ARRANGING VARIETY TRIALS INVOLVING A LARGE NUMBER OF VARIETIES. *Jour. Agr. Sci. [England]* 26: [424]-455, 1936.

## EXPERIMENTAL RESULTS

## EFFECT OF KERNEL SIZE UPON YIELD AND OTHER CHARACTERS

The average yield for the entire experiment was 42.7 bushels per acre, with a standard error for a single yield of 12.9 percent of this mean, using 8-foot rows. The effect of excess size of seed upon the yield was calculated from a comparison of the 2 sets of yields. If all yields are brought into the comparison, some are included which were secured from parent greenhouse seed with a kernel weight equal to or less than the kernel weight of the outside seed, as explained previously. Also, with the Ceres variety the crop was seriously affected with stem rust, which might easily nullify any parental effect of size of seed. In making the yield comparisons the square lattice lay-out was treated as an arrangement of ordinary randomized blocks or replications. When all the 288 samples are used in the analysis of variance, the yields from the seed maturing in the greenhouse and that maturing outside were 43.2 and 42.1 bushels, respectively. The difference required for significance is 1.4 bushel. With the omission of the Ceres variety and the 5 additional families, including Thatcher, the parent greenhouse kernels of which weighed less than, or essentially the same as, those of the outside seed, another calculation was carried through. The family 6.18-11.29 was retained, as it had suffered only moderate rust injury. The number of samples used was 192. The five families were omitted to allow a comparison between plants grown from seed clearly separated as to kernel size, grown in the greenhouse and out of doors. In this comparison seed from the greenhouse plants weighed 9.7 mg., or 31 percent, more per kernel than did that grown outside. The two yields, from the greenhouse and from the outside seed, are 45.33 and 43.36 bushels per acre, respectively, with a significant difference in yield of  $1.97 \pm 0.86$  bushels.

The correlation existing between the excess kernel weight of the greenhouse over the outside seed and the corresponding excess of yield was calculated, omitting the six families indicated above. The  $r$  value was found to be 0.08, which is not significant.

The means of various other characters calculated for the two parts of the experiment are to be found in table 5 along with differences and the difference necessary for significance.

TABLE 5.—Means of various characters calculated from 2 lots of wheat, planted from seed grown in the greenhouse and from seed grown out of doors, with differences and with differences necessary for significance

[96 pairs of parents are compared]

Character	Greenhouse	Nursery	Difference	Difference necessary for significance
Stand of seedling plant.....number.....	46.5	46.2	0.3	+0.5
Time of heading in June.....date.....	23.7	24.1	— .4	.3
Plants per row.....number.....	44.1	43.8	.3	.7
Heads per plant.....do.....	5.0	4.9	.1	.2
Fertile spikelets per head.....do.....	12.8	12.7	.1	.1
Sterile spikelets per head.....do.....	2.3	2.3	.0	.1
Kernels per head.....do.....	27.0	26.4	.6	.7
Spikelets with third kernel.....do.....	2.6	2.3	.3	.3
Weight of seed in 50 heads.....grams.....	46.6	45.9	.7	1.7
Weight of kernels.....milligrams.....	35.4	35.7	— .3	.7
Weight per bushel.....pounds.....	58.2	58.2	.0	.3
Weight of 36 straws.....grams.....	4.3	4.1	.2	.1
Yield per acre.....bushels.....	45.3	43.4	1.9	1.8

Two of the differences just reach the threshold of significance, while three are really significant. The difference in yield is indicated by odds of 47:1; the difference in date of heading has odds of 370:1; while for the weight of straw the difference has odds of 730:1.

#### A STUDY OF THE HYBRID GROUPS

In order to study the 18 families—the 16 hybrid families and the 2 varieties—the paired means of the various characters studied were combined. In table 6 these families are shown with their character means, their various rankings, the values of the significant differences, and their placements as to rank.

In the study discussed above the 144 pairs of selections were treated as a general population. With such diverse breeding as is indicated in table 1 it seems obvious that the families may properly be considered as units to be compared with one another.

The three wheats attacked by rust show relatively low yields. The low yield of Thatcher is less easily understood than that of Ceres and the hybrid family 6.18–11.29, even with Thatcher's incidence of leaf rust; and a discussion of its behavior will be considered later.

#### CALCULATION OF YIELDS

If the stand, the heads per plant, the kernels per head, and the weight per kernel are available, the yield per acre can be estimated. When this was done, using the data in table 6, the differences in yields obtained from threshing the crop and those derived by calculation averaged but  $0.3 \pm 0.14$  bushel. An average change of kernel weight of less than 0.1 percent would have resulted in essentially no difference between the two sets of yields. This close approximation is not surprising as the heads per plant and kernels per head were estimated from the results of the entire row. The weight per kernel was estimated from the sample from 50 heads.

#### CORRELATION AND REGRESSION STUDIES

Correlation coefficients were calculated, using the characters of yield, fertile heads per plants, kernels per head, and weight per kernel. This was done when the total of 288 units was used and also when the 3 low-yielding wheats, Thatcher, Ceres, and 6.18–11.29, were omitted, in which case there were 240 units used. These 3 wheats were all injured by rust. The kernel size of Thatcher was quite certainly reduced by leaf rust, and stem rust shriveled the kernels of the other 2. Partial coefficients were calculated in addition to the simple ones. The coefficients are given in table 7.

TABLE 6.—Means of the indicated characters obtained from seed matured in the greenhouse and in the open, grown in series 481 in 1938

[The hybrid selections from greenhouse and outside seed are here averaged to form the 18 family units]

No.	Key	Hybrid or variety	Yield per acre		Weight of seed 50 heads		Weight of 25 straws		Weight of 1,000 kernels		Weight per bushel		Fertile spikelets per head		Sterile spikelets per head		Heads per plant		Kernels per head		Spikelets with third kernel		Stand May 13		Days to heading in June	
			Bushels	Difference	(Grams)	Rank	(Grams)	Rank	(Grams)	Rank	Pounds	Rank	Number	Rank	Number	Rank	Number	Rank	Number	Rank	Number	Rank	Number	Rank	Average	Rank
1	23	3.2,3.85	51.8	0.7	45.7	8	4.12	13	34.8	13	88.4	8	12.7	11	2.4	16	5.5	3	26.3	1	2.9	3	46.9	4	23.0	5
2	35	6.19.6	51.1	1.3	50.7	1	4.34	6	36.7	3	55.8	17	12.9	2	2.3	10	5.7	1	25.6	15	1.8	12	46.5	9	23.0	6
3	21	3.2,3.67	49.8	2.8	44.4	11	4.51	1	35.5	10	57.3	13	12.6	13	2.3	11	5.5	4	27.0	5	9	16	46.5	13	24.3	15
4	15	3.2,3.64	47.0	4	45.3	10	4.27	8	36.7	4	58.3	9	12.7	8	2.0	4	5.0	8	26.7	9	2.2	9	46.6	7	22.4	2
5	55	6.19.9.29	46.9	5	47.8	5	4.24	11	37.1	2	58.5	5	12.7	10	2.2	8	5.0	9	26.8	8	2.5	7	46.7	5	22.4	10
6	31	5.7	46.5	1	44.0	12	4.36	4	34.2	15	65.7	3	12.7	10	2.2	8	5.1	6	28.1	2	2.7	6	46.3	10	24.1	13
7	51	6.19.6.29	45.2	3	45.3	3	4.27	9	36.6	6	57.9	12	12.7	5	2.3	12	4.9	10	26.5	10	2.9	4	45.6	14	22.5	3
8	41	6.19.7	45.2	3	45.3	3	4.27	9	36.6	6	57.9	12	12.7	5	2.3	12	4.9	10	26.5	10	2.9	4	45.6	14	22.5	3
9	11	3.2,3.30	44.4	6	43.4	14	4.19	14	33.3	11	68.1	1	13.1	1	2.3	13	5.1	7	27.0	6	3.0	2	45.1	18	24.1	12
10	53	6.19.7.29	44.4	6	43.4	14	4.19	14	33.3	11	68.1	1	13.1	1	2.3	13	5.1	7	27.0	6	3.0	2	45.1	18	24.1	12
11	45	6.19.0	43.1	1.3	44.3	12	4.31	7	38.3	14	68.0	10	12.9	4	2.3	13	4.8	13	25.3	18	3.3	1	46.6	11	23.2	7
12	43	6.19.9	42.9	2	44.3	12	4.31	7	38.3	14	68.0	10	12.9	4	2.3	13	4.8	13	25.3	18	3.3	1	46.6	11	23.2	7
13	13	3.2,3.33	42.5	4	47.8	6	4.23	12	35.9	9	57.1	14	12.7	7	2.2	7	4.9	12	27.7	7	2.2	10	46.7	8	23.6	8
14	33	5.9	40.4	2.1	45.5	9	4.26	10	35.1	12	58.6	6	12.7	10	2.2	7	4.9	12	27.7	7	2.2	10	46.7	8	23.6	8
15	61	6.19.0.29	39.5	5	48.3	4	4.36	5	36.3	8	59.4	7	12.7	9	2.3	15	4.7	18	23.4	11	5	18	47.0	3	26.3	18
16	63	6.18-11.29	34.5	5.0	34.3	16	3.70	16	28.8	17	55.8	15	12.5	14	2.1	16	4.4	18	23.4	11	5	18	47.0	3	26.3	18
17	25	Thatcher	28.8	3.1	23.7	17	2.96	18	20.6	17	55.8	15	11.6	18	1.8	17	4.2	19	23.2	12	1.2	15	46.4	16	22.6	4
18	65	Ceres	24.7	4.1	16.7	18	3.59	17	17.2	18	49.8	18	12.2	17	1.5	1	3.3	5	27.7	4	1.6	13	45.2	17	23.7	9
Mean of character			42.7	—	42.9	—	4.13	—	33.6	—	57.4	—	12.6	—	2.2	—	5.0	—	26.6	—	2.1	—	46.3	—	23.7	—
Significant differences			4.4	—	3.7	—	.32	—	2.0	—	.7	—	.35	—	.2	—	.5	—	1.6	—	.5	—	.38	—	.6	—
Fall between ranks <sup>1</sup>			34	—	78	—	1213	—	56	—	45	—	45	—	1112	—	56	—	910	—	56	—	32	—	132	—

<sup>1</sup> Except for yield, the values are not in order of size. The reading of % for example, indicates that all values below 47.5 g. of rank 7, are significantly lower than the high value of 50.7 g.

TABLE 7.—Simple and partial correlation coefficients of 15 hybrid families of wheat and also these plus Thatcher, Ceres, and 6.18-11.29

Characters involved <sup>1</sup>	Correlation coefficient for—		Characters involved <sup>1</sup>	Correlation coefficient for—	
	15 families	15 families plus Thatcher, Ceres, and 6.18-11.29		15 families	15 families plus Thatcher, Ceres, and 6.18-11.29
12.....	0.67	0.35	13.4.....	0.65	0.57
13.....	.50	.30	14.2.....	.36	.81
14.....	.08	.65	14.3.....	.26	.74
23.....	.07	.06	12.34.....	.86	.83
24.....	— .27	— .25	13.21.....	.80	.76
34.....	— .28	— .12	14.23.....	.70	.92
12.3.....	.74	.35	23.4.....	— .01	.04
12.4.....	.72	.70	24.3.....	— .26	— .25
13.2.....	.62	.36	34.2.....	— .27	— .10

<sup>1</sup> Code: 1, Yield; 2, heads per plant; 3, kernels per head; 4, weight per kernel.

Any coefficient in table 7 with a value of 0.25 or more is highly significant. The varieties Thatcher and Ceres have normally smaller kernel weights than components of the hybrid families. The kernel weights of these two varieties, and also of 6.18-11.29, were reduced from their normal by leaf rust or stem rust and yields were thus lowered. Because of this the correlation between yield and kernel weight for the 18 groups is decidedly higher than when these three units are omitted—the value of 0.65 compared with 0.08. The contrary is the case in the two correlations of yield and heads per plant for the Ceres and Thatcher varieties carried more heads per plant than the average. Also, the range in kernel weight is narrowed to about 4.1 mg., the maximum weight lying at 12 percent above the low value of 34.2 mg.

The correlation between heads per plant and weight of kernel is negative, —0.27, and highly significant. The values are essentially the same when Thatcher and Ceres are included. Removing Thatcher, Ceres, and 6.18-11.29, with their lightweight kernels, did not modify the correlation. This was evidently due in part to the fact that these wheats average high in heads per plant. The values are unchanged when kernels per head are held constant. The three characters primarily affecting yield do not have positive correlation with each other. Apparently it is by reason of this that the partial correlation coefficients between yield and the other characters take on such high values.

In the partial correlations calculated from the 15 families, those in which yield is correlated with heads per plant are larger than the other coefficients. It will be shown later in the discussion of partial regression coefficients that this relation was the most effective. A change of a unit in number of stools obviously affects the weight of grain per plant more than does a unit change in weight of kernel.

In the work by Bridgford and Hayes,<sup>10</sup> correlations similar to those considered here are given and a comparison will be of interest. Their terminology is modified to correspond to the writer's notation. Heads per row was used instead of heads per plant but the two characters probably correspond very well. In addition, Bridgford and Hayes used the three characters of plumpness (5), date of heading (7), and

<sup>10</sup> BRIDGFORD, R. O., and HAYES, H. K. CORRELATION OF FACTORS AFFECTING YIELD IN HARD RED SPRING WHEAT. Jour. Amer. Soc. Agron. 23: 106-117. 1931.

height of plant (8) in their calculations. These characters were not used in the work herein reported. The number of pairs used by Bridgford and Hayes in their correlation work was 61. The more interesting comparisons and values are shown in table 8.

TABLE 8.—*A comparison of partial correlation coefficients secured at Fargo, N. Dak., and by Bridgford and Hayes at Morris, Minn.*

Characters involved <sup>1</sup>	Values of Bridgford and Hayes <sup>2</sup>	Values in the present study based on —	
		15 families	18 families
12.34	0.62	0.86	0.83
13.24	.58	.80	.76
14.23	.56	.70	.92
12.34578	.02		
12.578	.04		
13.24578	.09		
13.578	.14		
14.23578	.22		
14.578	.23		

<sup>1</sup> Code: 1, Yield; 2, heads per row (or per plant); 3, kernels per head; 4, weight of kernel; 5, height of plant; 7, plumpness of kernel; 8, date of heading.

<sup>2</sup> These coefficients, except those of the fifth order, were calculated by the author from the zero-order coefficients of the original paper.

The coefficients from Bridgford and Hayes which can be compared with those of the present study, with two characters held constant, are uniformly lower. This may perhaps be attributed in part to the presumable lack of complete uniformity of stand in the Minnesota work with grain seeded thickly in rows. Compensations could not be made for differences in germination, for instance, which would likely be found from row to row when space-planting was not used. As has already been pointed out, the three characters, heads per plant, kernels per spike, and weight per kernel, are responsible for yield per plant; therefore, yield per unit area can be calculated when stand of plants is known. These three characters may be considered primary in determining yield while other characters are secondary in influence. Some other character may modify yield, such as date of heading, but only because one of the three primary characters mentioned is affected. The three coefficients from Bridgford and Hayes with five characters held constant are significantly lower than the corresponding coefficients with two of the primary yield characters held constant; for example, 0.02 is to be compared with 0.62. The mean of the coefficients when only the primary yield factors are concerned is 0.59 and only 0.11 when five factors are held constant. The correlation coefficient is calculated for yield and a factor primarily affecting yield while three factors secondarily affecting yield are held constant. This coefficient is then compared with one obtained when two factors primarily affecting yield are interposed into the group of three just indicated. The effect of this interposition is essentially nil. With the three secondary factors omitted from the group of five, the coefficient becomes highly significant.

Partial regression equations were calculated, two factors being held constant in each of the three cases. The regression coefficients and other values are shown in table 9.

TABLE 9.—Partial regression equations of yield on heads per plant, kernels per head, and weight per kernel, using 288 pairs of values with 18 families of wheat and 240 pairs with 15 families

[2 characters are held constant in each instance. Maximum and minimum yields and ranges are given corresponding to the extremes of the independent variables. All coefficients are highly significant.]

Basis of comparison and number of pairs	Regression of yield	X	Y	Difference in—	
				Heads per plant, kernels, or kernel weight	Bushels
Heads per plant:				<i>Number</i>	
240 .....	$Y = 7.43x_2 + 8.38$ .....	{ 4.4	{ 41.1	1.3	9.6
		{ 5.7	{ 50.7		
288 .....	$Y = 7.08x_2 + 7.20$ .....	{ 4.4	{ 38.4	1.3	9.2
		{ 5.7	{ 47.6		
Kernels per head:					
240 .....	$Y = 1.62x_3 + 2.35$ .....	{ 25.3	{ 43.3	3.0	4.9
		{ 28.3	{ 48.2		
288 .....	$Y = 1.58x_3 + 0.80$ .....	{ 25.3	{ 40.8	3.0	4.7
		{ 28.3	{ 45.5		
Weight per kernel:				<i>Milligrams</i>	
240 .....	$Y = 1.17x_4 + 3.26$ .....	{ 34.2	{ 43.3	4.1	4.8
		{ 38.3	{ 48.1		
288 .....	$Y = 1.18x_4 + 2.82$ .....	{ 17.2	{ 23.1	21.1	24.9
		{ 38.3	{ 48.0		

Like the coefficients of correlation shown in table 7, the regression coefficients with two characters held constant show no marked differences when comparisons are made between the 2 sets of families. Ceres and Thatcher and the family 6.18-11.29, all injured by rusts, were included in the 18-family group. The regression of yield on heads per plant is 7.43 bushels for the 15 families and this is significantly larger than the corresponding coefficient of 5.46 bushels. It is evident that an increment of 1 head per plant would modify the yield more than an increment of 1 mg. in kernel weight, and this fact is expressed in the relative size of the coefficients. It is obvious that the marked regression in yield on kernel weight in the one instance is due to the low kernel weight of Ceres, Thatcher, and 6.18-11.29. The predicted increase of 24.9 bushels would have been less with a normal development of the Thatcher kernel, but it likely would have remained of considerable moment.

From various estimates it was thought that the Thatcher yields in other experiments in 1938 at the North Dakota Agricultural Experiment Station and elsewhere were not cut much more than 25 percent by leaf rust; but, if this was true in the present experiment, the proper yield of Thatcher would have been between 37 and 38 bushels, which is still significantly below that of the better yielding wheats. It is doubtful whether leaf rust lessened the number of kernels per head, as there were five families not affected appreciably by leaf rust that had fewer kernels per head than Thatcher.

The weight of kernel of Thatcher has been obtained in 71 experiments from 1937 to 1939 at various points in North Dakota. In 5 of these the kernel weight was as high as 28 mg. and averaged 28.7 mg., the same as the kernel weight of 6.18-11.29. If the Thatcher kernel in the present experiment had had this weight, the yield would have been estimated at 39.7 bushels per acre, which is 11.2 bushels per acre below the average of the 3 highest yielding families. As the number of kernels per head for Thatcher is but 0.8 below the average of the



3 highest wheats, this factor does not explain the low yield for the variety.

As the difference between the top-yielding families and Thatcher seems to be greater than might be expected under field conditions, even with the modification suggested, the stand of plants may be considered as being below the level necessary for an optimum yield for the variety. If the Thatcher yield were to be readjusted to equal the maximum yields, its stand would be increased by nearly 30 percent to result in about 62 plants per row instead of the 48 used. If such an increase in stand were to result in a direct increase in yield, the plants could not suffer any decrease in heads per plant, although such a decrease would certainly arise if the stand increase were to be continued. The question arises, if the stand of Thatcher had been such as to produce its maximum yield, assuming its yield was not maximum, would the yields of the 3 top families have been augmented through a corresponding increase in their rate of seeding? Do the families with the high yields shown in table 6 represent the absolute maxima for the environment of 1938? They probably do not, and it remains a moot point whether the relatively low average yield of Thatcher in this experiment, brought about in part, as we have seen, by low kernel weight, could have been made equal to the high yields of some of the families by a sufficiently thicker planting.

The multiple-regression equation of estimate was calculated. For the 240 pairs this is—

$$Y = 7.43 \text{ heads per plant} + 1.62 \text{ kernels per head} + 1.17 \text{ kernel weight} - 76.8.$$

If one supplies the minimum and maximum family means, the extremes in yield become 36.9 and 56.2 bushels per acre. The smaller yield is but 2.6 bushels below the mean yield of family 6.19.0.29, while the larger yield is 4.4 bushels above the mean of the high-yielding family 3.2.3.85.

#### DISCUSSION

In the first experiment, carried out in 1936, it was shown that yields were increased by 10 or 12 percent when seed of heavy kernel weight was used in comparison with those from lightweight kernels. In the experiment of 1938 the heavy-kernelled seed resulted in an increased yield of but 4.5 percent, which was a significant value. In the first experiment the heavy kernels outweighed the lighter kernels by 50 percent and in the second by 31 percent. The greater percentage difference in yield in the first experiment must have been due in part to the greater disparity in size of seed. But it is possible that the severity of the season of 1936 might have played some part in emphasizing the lower yields from the lighter kernels. However the differences in the two experiments are rather small and must be considered in connection with their standard errors. Such small differences partake of significance only when many replications enter into the means. It is interesting that in the experiment of 1938 the measurement of two characters taken early, completeness of stand and earliness of heading, showed smaller differences in respect to the standard error than did the yield. One might expect the contrary to hold, under ordinary weather conditions; and Mallach<sup>11</sup> cites

<sup>11</sup> MALLACH, JOSEF. See footnote 2.

several workers who reported that plants grown comparably from heavy and from light seeds and showing measurable differences in size when young tended to lose this difference as they approached maturity.

In the experiment of 1938 the selections within a family or variety were combined to allow a study of yield as modified by characters of the plant. While size of kernel used in planting had at best only a minor effect upon yield, kernel size of the harvested plant did have a very significant influence. The two varieties Ceres and Thatcher and a hybrid family had relatively low yields because of small kernels. The kernels of Ceres and Thatcher are normally of lighter weight than those of the hybrid families, and rust attacks increased the normal disparity in weight. It was shown that even if the Thatcher variety had had a kernel weight of 28 mg. which is relatively high for that variety, the resulting yield would have been equivalent to about 39 bushels per acre, and still very significantly below the average of the hybrid families. The partial regression coefficient expresses more technically what has already been shown, that kernel weight of the harvested crop is a very important factor in influencing yield in this space-planted experiment. If leaf rust had not injured the Thatcher plant, more light would have been thrown upon the question as to whether a small-kerneled wheat plant can compete successfully in yield with one having large kernels when space planting is used. Reference to table 6 shows that the better yielding families are in general above the average for the three characters, heads per plant, kernels per head, and weight per kernel. This would be expected, from the relation of these characters to yield.

The 3 high-yielding families of table 6 had means for the 3 characters just mentioned of 0.6, 0.4, and -0.3 above or below the respective means of the 15 hybrid families. When these differences are applied to the 3 partial regression coefficients applicable to the 15 families, the 2 advances in yield are 4.5 bushels per acre for the excess in heads per plant, 0.6 bushel for kernels per head, and 0.4 bushel decrease for weight per kernel. Thus certainly some of the high yields shown in table 6 may be ascribed mainly to relatively heavy stooling, which also characterized Thatcher, and Ceres to a lesser degree. A kernel weight for Thatcher of 28 mg. is about as high as could be expected, a kernel weight which would place the Thatcher yield well below the average for the 18 groups. Thus, as was indicated earlier in the paper, an increased stand would be necessary to bring the yield of Thatcher up to the higher levels of yield. And here 2 things must be considered. Would the Thatcher plants in a theoretically optimum stand, or one increased by perhaps 30 per cent over that actually obtained, carry as many heads per plant as did the plants produced in the nursery? They probably would not. Would the high-yielding families seeded at correspondingly higher rates show correspondingly higher yields? Only when these questions are answered will it be possible to learn the maximum yielding capacities of the 2 classes of wheat, small-kerneled and large-kerneled, with approximately the same stooling capacity, when grown space-planted. To enhance the comparison, plantings could be made also at field rates. More satisfactory conclusions might then be obtained.

While it was shown that the three characters, heads per plant,

kernels per head, and weight per kernel, with a uniform stand are responsible for nearly all of the variability associated with yield under the conditions of the experiment, this is really not the end of the problem. While yield is important in the choice of a wheat variety, other characters must be taken into account, such as quality and relationship to disease other than stem and leaf rust, which were considered here. The character, strength of straw, has not been discussed as it did not enter into this work, but it is of importance in the choice of a variety. Obviously it should be possible to bring out a group of promising hybrid varieties but with differences among them similar to the differences found in table 6. Discussions similar to those in this paper would then be quite pertinent and would not be subject to the restrictions suggested above. The question could still be raised as to the extent to which the partial regression coefficients calculated from the spaced plantings could be applied to wheats planted at field rates. An experiment with this purpose should determine the differences in yielding capacity between large- and small-kerneled wheats. The value of such an experiment can scarcely be questioned.

#### SUMMARY

Seed wheat from a presumably pure line was separated into two classes with kernels weighing 26.6 and 40 mg. When comparisons were made between the yields of the two classes seeded by using either the same weight of grain or the same number of kernels per unit area, the heavier kernels showed the larger yield, the average differences being highly significant.

In a second experiment heavy-kerneled seed matured in the greenhouse was grown in comparison with lightweight seed matured out of doors. When only the 12 families which had parent greenhouse seed with kernels heavier than the outside seed were used, the average yield of grain from the heavy-kerneled seed was 1.97 bushels per acre above the yield from the seed maturing outside. This difference was significant and was 4.5 percent above the lower yield. The kernels of the greenhouse seed used in planting weighed 40.7 gm. per 1,000, or 31 percent heavier than the outside seed.

Correlation studies between the three independent characters, heads per plant, kernels per head, and weight per kernel, and the dependent character, yield, showed highly significant zero-order coefficients, except between kernel weight and yield when the three families injured by rust were omitted. All partial correlations of the first and second order were highly significant when yield was used as the dependent variable.

Partial regression coefficients were all highly significant. The regression of yield on heads per plant was nearly 7.4 bushels per acre for each unit change of head, 1.6 bushels for each unit change in kernels per head, and 1.2 bushels for each milligram change in kernel weight in the 15 families not injured by rust.

# INFLUENCE OF APHID RESISTANCE IN PEAS UPON APHID DEVELOPMENT, REPRODUCTION, AND LONGEVITY<sup>1</sup>

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## INTRODUCTION

The occurrence of aphid resistance in peas (*Pisum sativum* L.) was demonstrated by Searls<sup>3</sup> in 1931 and corroborated by Maltais<sup>4</sup> in 1936. Experiments of both workers were conducted under field conditions. Searls found that aphid numbers increased less rapidly on the Yellow Admiral and Onward varieties than on other strains of peas. He classified such varieties as "uncongenial" or "resistant." His conclusions were based on differences between seasonal curves of aphid increase on the varieties tested. These curves were plotted from data collected by making periodic aphid counts on random plant samples throughout the growing season. Maltais, using the same procedure, tested 21 standard pea varieties over a period of 3 years. He found, also, that varieties differed with respect to velocity of aphid increase, and selected Melting Sugar, Champion of England, and Prince of Wales as the most resistant of those studied. Yellow Admiral and Onward, unfortunately, were not included in these tests.

Plant breeders have become interested in recent years in the possibility of developing new plant varieties resistant to insect attack. Work on aphid resistance in peas has been delayed, however, due to the lack of a simple technique for measuring the resistance of plants within segregating families. The field testing procedure used by Searls and Maltais, while accurate, is not well adapted for use in a breeding program because of the large amount of time and labor required for a reading. It has been found, also, that field data based on yield or on the ability of plants to recover after aphid attack cannot be used safely to measure aphid resistance because of differences in the physiological age of plants at the time of aphid injury.<sup>5</sup>

The present study was begun in 1938 in an effort to learn more concerning the relationship between the pea plant and its aphid enemy. It was hoped that the results might aid in the development of a simple testing procedure of use to plant breeders. This paper presents data secured from two experiments concerned with the influence of aphid resistance in peas upon aphid development, reproduction, and longevity.

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<sup>3</sup> SEARLS, ED. M. A PRELIMINARY REPORT ON THE RESISTANCE OF CERTAIN LEGUMES TO CERTAIN HOMOPTEROUS INSECTS. *Jour. Econ. Ent.* 25: 46-49. 1932.

<sup>4</sup> MALTAIS, J. B. RESISTANCE OF SOME VARIETIES OF PEAS TO THE PEA APHID *ILINOIA PISI KALT*. *Ent. Soc. Ont. Ann. Rpt.* (1936) 67: 40-45, illus. 1937.

<sup>5</sup> HARRINGTON, CECIL D., and SEARLS, ED. M. INFLUENCE OF THE PHYSIOLOGICAL AGE OF THE PEA PLANT ON ITS RECOVERY FROM APHID DAMAGE. *Jour. Agr. Res.* 60: 157-161, illus. 1940.

## PROCEDURE

Two varieties of canning peas, Perfection and Pride, were used in the study. The former was selected because of its susceptibility to the pea aphid, the latter because of its partial resistance. Plants were grown in sand, using a complete nutrient solution developed for peas by the Plant Pathology Department of the University. The containers were No. 2. enameled tin cans. The aphids (*Macrosiphum (Illinoia) pisi* (Kleb.)) were of pure-line origin, the line being obtained by isolating a single agamic insect and its subsequent progeny.<sup>6</sup> Gauze-covered lantern globes were used to confine the insects to the growing tips of the test plants. Support for these globes, as well as for the plants



FIGURE 1.—Testing racks with plants and lantern globes in position.

and their containers, was provided by adjustable testing racks, as shown in figure 1.

The globes were ventilated by electric fans located at the front and along the sides of the testing bench. Currents of air directed over the gauze-covered tops of the globes created a partial vacuum within. This caused fresh air to circulate through screen-covered apertures in the platform upon which each globe rested.

Each experiment included 30 Perfection and 30 Pride plants. Each of these plants was infested with a single reproducing aphid. After 4 hours, however, this aphid and all but one of its progeny were removed. Plants on which no young were produced during this short period were infested with one of the excess young removed from other plants. In

<sup>6</sup> Little evidence has appeared to date which would indicate that more than one strain of the pea aphid exists. Nevertheless, to avoid any genetic heterogeneity which might be present, pure-line insects were used.

this way 60 aphids of uniform age were confined upon the 30 individual plant tips of the two varieties.

Data were taken at 4-hour intervals during the period of aphid development. All insects were examined carefully with a hand lens at each reading to determine whether a new stadium had been reached during the previous 4-hour period. This procedure enabled data to be taken relative to the number of hours required by each of the 60 aphids to pass through each of the four nymphal stadia and to reach reproductive maturity.<sup>7</sup> After reproduction began, the progeny of each aphid was carefully removed and counted every 48 hours. The young were removed to prevent overcrowding of the test plants, and to avoid second-generation reproduction which would have resulted if any of the progeny had been allowed to reach maturity. When emaciation and reduced reproduction indicated that the parent aphids were growing weaker, observations were begun and continued at 24-hour intervals in order to record the day on which each parent aphid died. The experiments, which ran consecutively, were concluded upon the death of the last parent aphid in each. Individual records were kept, all data pertaining to any aphid being recorded under its respective rack and globe number.

#### EXPERIMENTAL RESULTS

A complete set of data was obtained for the second experiment. All averages, for this reason, are based on the full complement of 30 replicates per variety. While taking data on reproduction in the first experiment, however, 8 parent aphids were dislodged and lost. In addition, failure to find and remove all young resulted in second generation reproduction on 3 plants. Data relative to total reproduction were discarded in these cases. No records of longevity could be obtained for the 8 lost aphids. Furthermore, longevity records for 3 other aphids were discarded because the insects died of causes other than old age. In the first experiment, therefore, the averages of total reproduction for Pride and Perfection are based on records of 24 and 25 aphids, respectively, and the averages of total longevity are based on longevity records of 26 and 23 aphids, respectively. The data are presented in table 1.

#### STATISTICAL TREATMENT AND INTERPRETATION OF DATA

"Student's"  $t$ -test was used in the statistical treatment of the data, the values of  $t$  (for 58 degrees of freedom) necessary for significance at the 0.05 and 0.01 levels being 2.002 and 2.664, respectively.

It appears from the two experiments that aphid development on Pride differed significantly from that on Perfection with regard to hours to first moult. The aphids required a greater number of hours on Pride, the values of  $t$  being significant at the 1 percent level. This difference persists, as far as time is concerned, to the second, third, and fourth moults, the respective  $t$  values all being positive and significant. Under these circumstances a difference is anticipated in total time required from birth to reproductive maturity, with aphids on Pride requiring the greater length of time. This hypothesis is supported by a positive value of  $t$ , also significant at the 1 percent level.

<sup>7</sup> The pea aphid, theoretically, is mature as soon as it reaches the last, or fifth, stadium. The insects however, do not begin to reproduce until sometime later.

The length of the first stadium is the time required to reach the first moult. There is a significant difference between varieties in the first experiment with regard to the time spent in the second stadium and in the period between the last moult and reproductive maturity, the two *t* values being significant at the 5 percent level. Except for these differences, in both experiments the original variation between Pride and Perfection, observed in the time to the first moult, remained essentially the same from then on. In other words, there were no significant differences in time spent in the second, third, and fourth stadia, and in the period just prior to reproductive maturity.

TABLE 1.—*Aphid development, reproduction, and longevity on the Perfection and Pride varieties*

Item	Development period (average hours)										Total young produced	Total life span
	Length of stadia				Fourth moult to reproductive maturity <sup>1</sup>	Period from birth to various moults				Birth to reproductive maturity <sup>2</sup>		
	First	Second	Third	Fourth		First	Second	Third	Fourth			
Experiment 1:	<i>Hours</i>	<i>Hours</i>	<i>Hours</i>	<i>Hours</i>	<i>Hours</i>	<i>Hours</i>	<i>Hours</i>	<i>Hours</i>	<i>Hours</i>	<i>Hours</i>	<i>Number</i>	<i>Days</i>
Perfection	35.0	36.6	40.9	45.3	20.6	35.0	71.5	112.4	157.7	178.3	106.4	29.0
Pride	38.2	38.2	40.9	44.9	22.4	38.2	76.4	117.3	162.2	184.6	93.5	23.9
Difference	3.2	1.6	.0	— .4	1.8	3.2	5.1	4.9	6.5	6.3	— 12.9	— 5.1
<i>t</i> Value <sup>3</sup>	**5.167	*2.548	.000	.491	*2.032	**5.167	**5.605	**4.705	**4.191	**5.754	**4.435	**5.070
Experiment 2:	<i>Hours</i>	<i>Hours</i>	<i>Hours</i>	<i>Hours</i>	<i>Hours</i>	<i>Hours</i>	<i>Hours</i>	<i>Hours</i>	<i>Hours</i>	<i>Hours</i>	<i>Number</i>	<i>Days</i>
Perfection	33.0	38.6	40.0	51.1	19.8	33.0	71.5	111.5	162.6	182.5	109.7	32.6
Pride	35.0	39.2	40.0	52.8	20.4	35.0	74.2	114.2	167.0	187.4	95.6	25.3
Difference	2.0	.6	.0	1.7	.6	2.0	2.6	2.7	4.4	4.9	— 14.1	— 7.3
<i>t</i> Value <sup>3</sup>	**3.042	1.167	.000	1.545	.784	**3.042	**4.882	**3.466	**3.084	**3.609	**4.271	**5.470

<sup>1</sup> Total hours between fourth moult and reproductive maturity.

<sup>2</sup> Total hours between birth and reproductive maturity.

<sup>3</sup> For 58 degrees of freedom, *t* at the 0.05 level = 2.002, and at the 0.01 level = 2.664.

\*Indicates a significant difference exists at the 0.05 level. (Odds are 20-1 that difference is not due to chance alone).

\*\*Indicates a significant difference exists at the 0.01 level. Odds are 100-1 that difference is not due to chance alone.

Fewer young were produced on Pride than on Perfection, the difference being significant at the 1 percent level. The total length of life was significantly less on Pride, the *t* value being significant at the 1 percent level.

A study of data on aphid development brings to light some interesting information regarding the influence of resistance upon aphids reared on Pride as compared with those reared on Perfection. This influence may be described as follows: Resistance slows down the rate of development in all stadia, but the reduction is usually too small to be detected statistically for any one stadium, except the first. The influence is, however, cumulative, increasing gradually until reproductive maturity is reached. This trend is particularly noticeable in the results of the second experiment.

## DISCUSSION

Under Wisconsin conditions the pea aphid normally produces 15 to 16 generations each year, which are roughly distributed as follows: 2 to 3 upon the primary hosts (alfalfa) in the spring, 7 to 8 upon secondary hosts (peas) in late spring and summer, and 3 upon the primary hosts again in the fall. Peas are planted in April or early May and are

harvested the last week of June (early varieties) or in early July (late varieties). One aphid generation requires 6 to 12 days depending upon the temperature. When peas are young, low temperatures inhibit aphid development and reproduction with the result that little damage is done. With the higher temperatures of late June, aphid generations follow each other in rapid succession, the population soon reaching sufficient proportions to blast blossoms and young pods or even to destroy the plants. The three principal factors that determine whether a pea field is to be damaged are: (1) Size of original infestation, (2) rate of aphid development and reproduction, and (3) duration of infestation. The grower cannot control the original infestation but use of resistant varieties will slow down the rates of aphid development and reproduction. While it is true that the most resistant varieties known today are but mildly resistant, nevertheless the inhibiting influence that they exert upon aphid build-up during the entire season is very large. In this connection the data presented above can be considered. By computing the differences between *Pride* and *Perfection* on the percentage basis, utilizing the data in the three right-hand columns of table 1, it is found that *Pride* in the two experiments increased the time of development 3.1 percent, decreased reproduction 12.5 percent, and shortened the aphid life span by 20 percent. The cumulative effect of this influence, acting upon 8 successive aphid generations, may explain why the more resistant varieties often produce a crop when susceptible strains succumb.

Lack of a simple and accurate technique for determining the comparative resistance of segregating plants is one of the chief factors which has delayed the production of new pea varieties resistant to the pea aphid. The principal purpose of this work, as mentioned above, was to discover which component of the host-parasite complex could be used to best advantage as a basis for measuring the relative aphid resistance of pea plants. In this respect the data indicate that the 3.1 percent increase in time of aphid development for *Pride* as compared with that for *Perfection* is not large enough to justify basing a technique on this phase of the life cycle. This fact becomes clear when it is realized that the known range from maximum resistance to maximum susceptibility in pea varieties tested to date is very small, *Pride* and *Perfection* representing almost the extremes. The difference of 12.5 percent in reproduction between the two varieties appears to be large enough to justify further work on this subject. The 20 percent difference in longevity is promising, but unfortunately the use of longevity data in measurement of aphid resistance is out of the question due to (1) the technical difficulty of caring for the progenies, and (2) the long period of time (during which a test must be run) before the test insects die.

#### SUMMARY AND CONCLUSIONS

Two experiments were run under greenhouse conditions to determine the influence of aphid resistance in peas upon aphid development, reproduction, and longevity. The partially resistant *Pride* and the susceptible *Perfection* pea varieties were used. Both experiments include 60 aphids, which were confined individually upon 30 plants of each variety. Analysis of the data obtained leads to the following



conclusions, which should be considered only with reference to the varieties used:

1. Aphid resistance reduces slightly (3.1 percent) the rate of aphid development.
2. Aphid resistance inhibits reproduction to a considerable extent (12.5 percent).
3. Aphid resistance decreases aphid longevity (20 percent).
4. The cumulative effect of resistance acting upon aphid populations throughout the pea growing season may explain why resistant varieties are often able to produce a crop when susceptible strains are destroyed.
5. Data on relative rates of aphid reproduction appear to offer a better possibility for measuring the comparative resistance of pea varieties than data on rates of aphid development or length of life.

# BUCKEYE ROT OF TOMATO IN CALIFORNIA<sup>1</sup>

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## INTRODUCTION

Although buckeye rot of green tomato fruits (*Lycopersicum esculentum* Mill. var. *vulgare* Bailey), caused by *Phytophthora terrestris*, was described by Sherbakoff (25)<sup>2</sup> more than two decades ago, the disease was not known to occur in California, where tomatoes are a major crop, until September 1937 when it was observed by J. B. Kendrick in a 100-acre tomato field near Davis. The rot was confined to fruits on or near the ground following a heavy irrigation. In August 1938, infected green Italian pear-shaped tomatoes were received from Modesto, and the disease, based on symptoms only, was identified as buckeye rot. Two months later, this disease was found to be of major importance in the late crop of green-wrap tomatoes grown at Brentwood.

Buckeye rot of green tomato fruits has consistently been ascribed to *Phytophthora parasitica* by investigators in various states and foreign countries. Some of the reported diagnoses were made, apparently, on symptomatology alone or at least on very limited cultural tests. However, Bewley (4), in England, may have been the first to suggest that more than one species of *Phytophthora* may cause buckeye rot, naming *P. cryptogea* in addition to *P. parasitica*. Investigations in California not only support Bewley's contentions, but, in addition, indicate that the disease may be produced by other species in the absence of *P. parasitica*.

## DISTRIBUTION OF THE DISEASE

According to Sherbakoff (25, 26, 27), buckeye rot of tomato fruits was first recognized as a new disease in January 1915 at Goulds, Fla., but presumably had been observed as early as 1911, and occurs along both the east and west coasts. The disease has been reported to the Division of Mycology and Disease Survey, United States Department of Agriculture,<sup>3</sup> from Arizona, Arkansas, Colorado, Florida, Illinois, Indiana, Louisiana, Maryland, Massachusetts, Mississippi, Missouri, New Jersey, New York, North Carolina, Ohio, Tennessee, Texas, Virginia, and West Virginia. It also occurs in Puerto Rico. The first record of the disease from California was given by Ramsey<sup>4</sup> in 1939. In view of its wide geographical range, Weimer (39) was undoubtedly correct in stating that "buckeye rot of tomatoes, then, is not restricted to the extreme southern States."

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<sup>2</sup> Italic numbers in parentheses refer to Literature Cited, p. 472.

<sup>3</sup> Letter from H. A. Edson, Division of Mycology and Disease Survey, U. S. Department of Agriculture, dated February 19, 1940.

<sup>4</sup> RAMSEY, G. B. FRUIT AND VEGETABLE DISEASES ON THE CHICAGO MARKET IN 1938. U. S. Dept. Agr. Bur. Plant Indus., Plant Disease Rptr., Sup. 114, pp. [27]-40. 1939. [Mimeographed.]

The disease has also been reported in Argentina (5), Australia (14), the British West Indies (Trinidad, St. Vincent, and Montserrat) (1), Canada (7), Denmark (10, 37), England (2, 3, 4, 15, 16, 17), India (33), Japan (29), Mexico (28), the Netherlands (18), Palestine (20), and the Union of South Africa (34, 35).

Further records, which may possibly relate to buckeye rot, occur in the literature and are briefly mentioned in order to make this paper as inclusive as possible. A rot of green tomato fruits, presumably caused by *Phytophthora terrestris*, was found in Cuba in 1918 by Bruner, according to Weimer (39). In 1932, just 7 years prior to the first definite record of the disease in the British West Indies (1), Briant (6) found that an unnamed species of *Phytophthora* was responsible for a fruit rot of tomatoes in Trinidad. In Bermuda, a species of *Phytophthora* belonging to the *P. palmivora* group, was believed by Waterston (36) to be the cause of a rot of green tomato fruits (varieties Marglobe, Pritchard, and Break o'Day).

Although the disease has not been reported from Greece and Italy, nevertheless it is significant that Sarejanni (24) and Goidanich (9) have recently described a collar rot of tomato plants caused by *Phytophthora parasitica*. Samuel (22) stated the disease has never been observed in South Australia, although the disease is known to occur in the metropolitan area of Sydney, Australia (14).

A rot of tomato fruits, caused by *Phytophthora capsici*, has occurred in the Arkansas Valley of Colorado (23), but whether the disease is identical with buckeye rot was not indicated. <sup>5</sup>

#### SYMPTOMS OF THE DISEASE

The symptoms of buckeye rot of green tomato fruits collected in the Brentwood and Stockton sections, two of the four known centers of infestation in California, are identical with the original description as given by Sherbakoff (25, 26, 27) and later by Kendrick (11), Ramsey and Link (19) whose paper is illustrated by excellent colored photographs, Wager (34, 35), Weber and Ramsey (38), and Young, Harrison, and Altstatt (40). Green fruits of all sizes, if in contact with or near the surface of moist or waterlogged soil, were subject to infection. Irregular-shaped, water-soaked areas, usually but not always at the blossom end of the fruits, constituted the first visible evidence of infection (fig. 1, A). As the lesions enlarged, the centers turned brown to blackish-brown and frequently developed either complete or incomplete concentric brown rings (fig. 1, B, D). Some fruits were devoid of this ring symptom. On all lesions, however, the advancing margin was water-soaked, while the rate of enlargement was rapid under conditions of high temperature and humidity and an excessive supply of irrigation water. Decayed areas were firm in texture, with little or no aerial mycelium on the surface of the fruits. An abundant mycelial growth developed, however, when diseased fruits were placed in a moist chamber, and the rate of decomposition was accelerated. Lesions on some fruits showed arrested development, the surface tissues becoming hard and dry (fig. 1, C).

<sup>5</sup> Since this paper was accepted for publication the following article has appeared: KREUTZER, W. A., BODINE, W. W., and DURRELL, L. W. CUCURBIT DISEASES AND ROT OF TOMATO FRUIT CAUSED BY *PHYTOPHTHORA CAPSICI*. *Phytopathology* 30: 972-976, illus. 1940. In this article a field decay of tomato fruits, caused by *Phytophthora capsici*, is described in more detail.

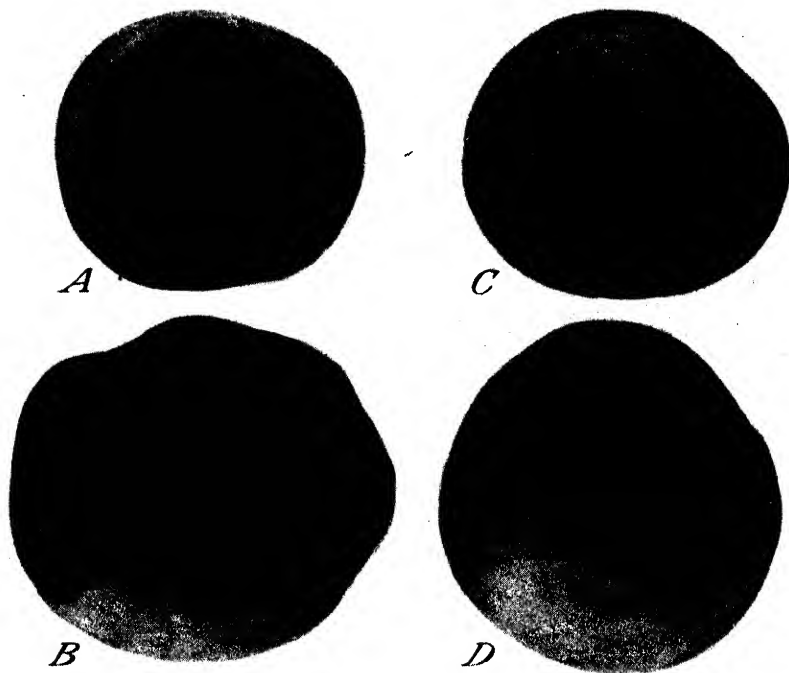


FIGURE 1.—Natural infection of green-wrap tomato fruits grown at Brentwood, Calif.: A, Large, irregular-shaped, water-soaked lesion, with brown center; B, an aggregate of brown lesions surrounded by a water-soaked margin; C, blackish-brown lesions showing arrested development; D, water-soaked lesion with brown center and concentric rings.

#### THE CAUSAL FUNGI, *PHYTOPHTHORA CAPSICI* AND *P. DRECHSLERI*

Small tissue fragments were removed aseptically from the advancing, internal margin of lesions of naturally infected, green tomato fruits, planted on malt-extract agar (13) in Petri dishes, and incubated at room temperature. Usually, after 48 hours, pure cultures were established on agar slants by transferring hyphal tips from the edge of the colonies. Cultures were made from approximately 300 green tomato fruits in 1938 and 1939.

Although the original cause of buckeye rot was ascribed by Sherbakoff (25) to *Phytophthora terrestria*, Tucker (32) later determined that the fungus was identical with *P. parasitica* Dast.

Microscopic examination of the California isolates indicated that two species of *Phytophthora* were involved in the rotting of green tomato fruits, namely, *P. capsici* Leonian (12) and *P. drechsleri* Tucker (30).<sup>6</sup> Cultures of *P. drechsleri* were more numerous than *P. capsici* in 1938, whereas in 1939 all the isolates were referable to *P. capsici*.

<sup>6</sup> An isolate from a rotting tomato fruit collected at Edison, Calif., in November 1940 was identified as *Phytophthora parasitica* Dast. The isolate developed the tufted type of mycelial growth characteristic of *P. parasitica*, formed sporangia and chlamydospores in culture, and exhibited temperature growth relations typical of the species. The isolate was indistinguishable from cultures obtained from tomato fruits from Cuba, Florida, and Missouri.

It is significant that not a single isolate of *P. parasitica* appeared during either season, although this species has heretofore been recognized as the exclusive causal agent of the disease.

The three species have similar temperature-growth relations and may be expected to appear on tomato fruits under similar environmental conditions. *Phytophthora drechsleri* is readily distinguishable from the other species by its nonpapillate sporangia which usually develop sparingly, even when hyphae are transferred to sterile distilled water. *P. capsici* does not develop typical chlamydospores and may thus be distinguished from *P. parasitica*. Furthermore, the sporangia of *P. capsici* are usually somewhat irregular, with a tendency to elongate in the apical region, while those of *P. parasitica* are more frequently of the regular, limoniform type. All three species are variable in production of oogonia, some isolates developing them in fair numbers while in others the sexual stage is never observed.

Tucker (32) has shown that the ability to invade and kill stems of pepper plants is a specific character of *Phytophthora capsici*. All isolates from tomato fruits that resembled *P. capsici* morphologically were inoculated into pepper stems; in every instance the fungus invaded and killed the terminal 3 to 6 inches of the stem in 5 days.

Pure cultures of *Phytophthora capsici* and *P. drechsleri*, isolated from naturally infected green tomato fruits collected at Brentwood, Calif., and of *P. parasitica* from an infected tomato fruit collected at Columbia, Mo., were used in the infection experiments. The fungi were grown on malt-extract agar in Petri dishes, incubated at room temperature, and used for inoculum when 4 days old.

Parallel inoculations were made on unwounded, detached, green tomato fruits in the laboratory and on green fruits in situ on healthy tomato plants grown in the greenhouse. In the laboratory, healthy fruits were washed in running tap water, rinsed in distilled water, and dried. A small block of inoculum was placed on the uninjured epidermis and kept moist with absorbent cotton under an inverted preparation dish. In some instances, inoculated fruits were placed in glass moist chambers. No fruits were wounded in any of the inoculation tests. For controls, fruits were handled in the same manner except that sterile agar was substituted for the inoculum.

In the greenhouse, the green fruits on living tomato plants, varieties Sutton's Best of All and Crackerjack, were not washed. The inoculum and moist absorbent cotton were held in place with a rubber band. One to several inoculated fruits were enclosed in a glassine bag after inoculation. A total of 12 fruits were inoculated with each species of *Phytophthora*. Control fruits on living plants were similarly treated except that sterile agar was substituted for the inoculum. All inoculated fruits became diseased, while the noninoculated control fruits remained healthy. The average incubation period was: *Phytophthora capsici*, 7 days; *P. drechsleri*, 11 days; and *P. parasitica*, 7½ days. These artificially induced lesions were identical in color and consistency with those resulting from natural infection. Concentric rings, either complete or incomplete, developed in some lesions (fig. 2, A), but usually this symptom was lacking (fig. 2, B). It is doubtful whether the zonation said to be characteristic of buckeye rot is a good distinguishing character. In these inoculation tests, it was observed that fruits vary a great deal in the development of the zonate

character, even when infected by the same isolate. The 3 species of *Phytophthora* produced identical symptoms when compared with each other, and it was impossible, therefore, to differentiate between them by using expressed symptoms as criteria. Laboratory tests yielded comparable results, with no essential difference in the incubation periods of the respective species tested. Reisolations were made from all infected fruits, and infection was again obtained with the reisolates.

These tests for pathogenicity indicate that species other than *Phytophthora parasitica* may also cause buckeye rot of tomato, and, in California, they are represented by *P. capsici* and *P. drechsleri*.

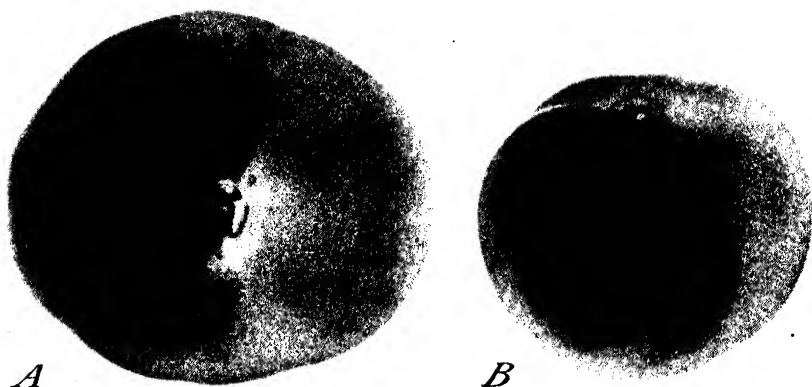


FIGURE 2.-- Artificial infection, without wounding, of green Crackerjack variety tomato fruits, in situ on plants grown in the greenhouse. A, Large, water-soaked lesion, with slightly darker center, showing concentric ring effect produced by *Phytophthora capsici*; B, large, water-soaked lesion, with blackish-brown center, produced by *P. drechsleri*.

An isolate of *P. capsici* from a naturally infected honeydew melon fruit (*Cucumis melo* L. var. *inodorus* Naud.) has previously been shown to be pathogenic to green tomato fruits (31), resulting in lesions typical of buckeye rot.

That green or immature tomato fruits are highly susceptible and may be invaded by *Phytophthora parasitica* in the absence of wounds was previously mentioned by Sherbakoff (25), Gardner (8), Kendrick (11), Ramsey and Link (19), Rosenbaum (21), Wager (35), and Weber and Ramsey (38), so that the results presented in this paper are merely confirmatory.

#### DIFFERENTIAL HOSTS

Parallel inoculations of various fruits, roots, and tubers were made in the laboratory with 4-day-old cultures of *Phytophthora capsici*, *P. drechsleri*, and *P. parasitica* in an attempt to find a differential host which could be used for rapid identification of various isolates from naturally infected green tomato fruits. Infection of ripe tomato, eggplant (*Solanum melongena* L. var. *esculentum* Nees), avocado (*Persea gratissima* Gaertn.), Bartlett pear (*Pyrus communis* L.), and honeydew melon fruits was obtained with the three species of *Phytophthora*, but all produced identical symptoms on these hosts.

However, Yellow Crookneck and Zucchini pumpkin fruits (*Cucurbita pepo* L. var. *condensa* Bailey) were readily infected by *Phytophthora capsici* and *P. parasitica*, but not by *P. drechsleri*; Purple Top White Globe turnip roots (*Brassica rapa* L.) were infected only by *P. drechsleri* and *P. parasitica*; and carrot roots (*Daucus carota* L. var. *sativa* DC.) by *P. capsici* and *P. drechsleri*. *P. capsici* alone caused infection of bell or sweet green pepper (*Capsicum annuum* L. var. *grossum* Sendt.), Newtown Pippin apple (*Pyrus malus* L.), and cucumber fruits (*Cucumis sativus* L.). Thus, for the isolates tested, pumpkin and bell pepper fruits and turnip and carrot roots may be used as differential hosts.

All three species of *Phytophthora* failed to infect Garnet Chili and White Rose potato tubers (*Solanum tuberosum* L.); muskmelon (*Cucumis melo* L.), Klondike watermelon (*Citrullus vulgaris* Schrad.), and lemon fruits (*Citrus limonia* Osbeck); and garden beet roots (*Beta vulgaris* L. var. *crassa* Alef.).

### SUMMARY

Buckeye rot of green tomato fruits is prevalent in several of the interior valleys of central California.

The disease is favored by high temperatures and humidity, and the most important factor favoring infection in California appears to be contact of the fruit with moist soil or with irrigation water.

Isolation, inoculation, and taxonomic studies show that the disease in California is caused by *Phytophthora capsici* and *P. drechsleri*, rather than by *P. parasitica*.

Several differential hosts, including pumpkin and bell pepper fruits and carrot and turnip roots, were found. These may assist in the identification of *Phytophthora* species isolated from diseased tomato fruits.

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# RING NECROSIS OF CABBAGE<sup>1</sup>

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## INTRODUCTION

In 1935 Smith (4)<sup>3</sup> described a virus disease of cabbage and brussels sprouts in England, which he designated as "ringspot" because of the characteristic deeply sunken lesions surrounded by almost black necrotic rings. When leaves of young cabbage plants were inoculated, necrosis usually developed on the inoculated leaves and a mosaic mottling without preliminary vein clearing developed in the younger systemically infected leaves. Necrotic and occasionally chlorotic rings followed. In 1938 Tompkins et al. (9) described a virosis of cabbage from California under the name of "black ring." The early symptoms on inoculated cabbage (no reaction on the inoculated leaves themselves was recorded) consisted of numerous small chlorotic lesions in which necrotic margins developed commonly after 2 or 3 weeks. Under field conditions the necrotic rings were most conspicuous on the under surface of the older, outer head leaves. It is to be noted that neither mosaic mottling nor vein clearing was described in connection with this disease on cabbage.

In 1937, at Madison, Wis., the writers noted in a plot of cabbage infected with a mosaic disease described elsewhere (2) necrotic ring lesions on many mature plants, particularly on the outer head leaves. From such plants a virus was obtained that produced various degrees of chlorosis and necrosis on cabbage. Although it had certain points in common with the diseases noted above, it was sufficiently distinct to warrant the present description. In order to distinguish the disease from others it is designated herein as "ring necrosis" of cabbage. A preliminary report has been published (3).

## SYMPTOMS AND HOST RANGE

One of the major points of difference between ring necrosis and black ring is the fact that ring necrosis is masked in the greenhouse at 13° to 19° C. The symptoms herein described were observed on plants growing at 22° to 25°.

## CRUCIFEROUS HOSTS

The following crucifers were inoculated mechanically with ring necrosis virus, according to methods described in the section on trans-

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<sup>3</sup> Italic numbers in parentheses refer to Literature Cited, p. 491.

mission of the virus, and all were found to be susceptible and to exhibit systemic symptoms.

*Brassica oleracea* var. *capitata* L. (cabbage, vars. Jersey Queen, Marion Market, Wisconsin All Seasons, Wisconsin Hollander).

*Brassica oleracea* var. *botrytis* L. (cauliflower, var. Snowball; sprouting broccoli, var. Green Sprouting).

*Brassica oleracea* var. *gongylodes* L. (kohlrabi, var. Early White Vienna).

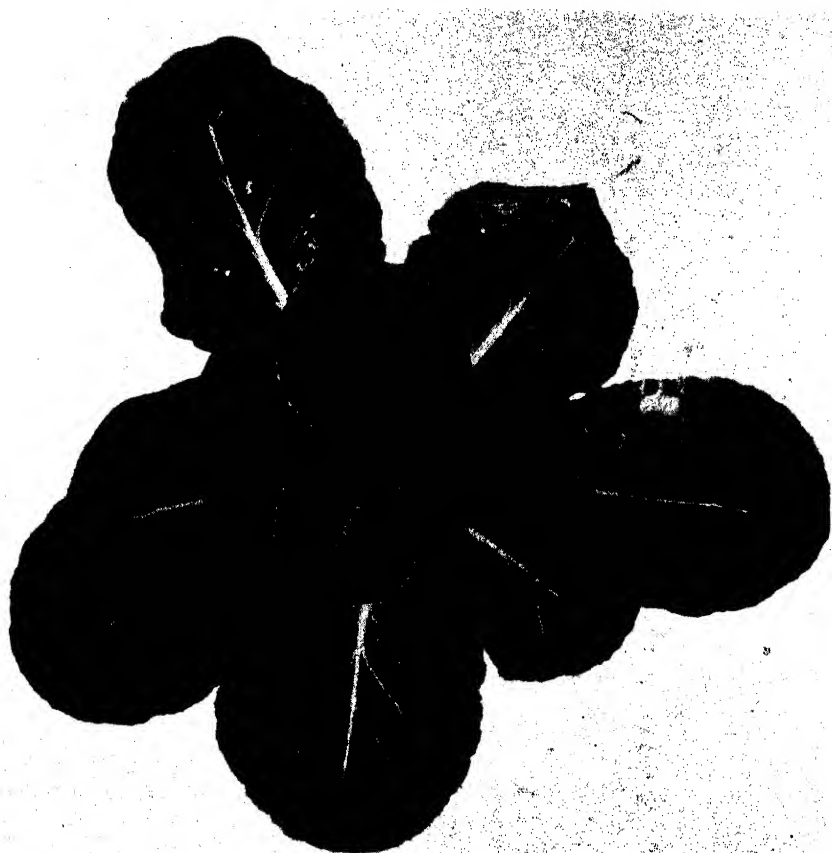


FIGURE 1.—Young cabbage plant infected with the ring necrosis virus, showing small chlorotic lesions on younger leaves and necrotic ring development on the more mature leaves.

*Brassica oleracea* var. *gemmifera* Zenker (brussels sprouts, var. Long Island Improved).

*Brassica oleracea* var. *viridis* L. (kale, var. Dwarf Green Curled).

*Brassica campestris* var. *napobrassica* DC. (rutabaga, var. American Purple Top).

*Brassica napus* L. (rape, var. Dwarf Essex).

*Brassica rapa* L. (turnip, var. Purple Top White Globe).

*Brassica juncea* (L.) Coss. (leaf mustard, var. Tender Green).

*Brassica pekinensis* (Lour.) Rupr. (Chinese cabbage, var. Chihli).

*Brassica hirta* Moench (*B. alba* (L.) Boiss, white mustard).

*Brassica nigra* (L.) Koch (black mustard).

*Cheiranthus allionii* Bailey (Siberian wallflower).

*Mathiola incana* var. *annua* (L.) Voss (annual stock, var. Dwarf Ten Weeks).  
*Hesperis matronalis* L. (dames rocket).  
*Raphanus sativus* L. (radish, var. French Breakfast).  
*Berteroa incana* DC. (hoary alyssum).  
*Capsella bursa-pastoris* (L.) Medic. (shepherds-purse).  
*Lepidium virginicum* L. (wild peppergrass).  
*Lepidium sativum* L. (garden cress).  
*Thlaspi arvense* L. (pennycress).  
*Neslia paniculata* (L.) Desv. (ballmustard).  
*Sisymbrium officinale* (L.) Scop. (hedgemustard).  
*Sisymbrium altissimum* L. (tumblemustard).

The symptoms on some of these hosts will be described.

#### CABBAGE

No symptoms appear on inoculated leaves of cabbage (*Brassica oleracea* var. *capitata*), but 17 to 21 days after inoculation small con-



FIGURE 2.—A cabbage leaf affected with ring necrosis. Note various stages of transition from the chlorotic spot through the development of a necrotic center and marginal ring to the final "bull's-eye" lesion.

spicuous yellow lesions appear in the parenchyma of the fourth or fifth unfolded leaf, at the distal portion from the growing tip (fig. 1). The more intense chlorosis at the outer margin of the lesions gives the appearance of a halo. Since the lesions are usually numerous they

may impart collectively the effect of mottling, but there is neither true interveinal mosaic nor systemic vein clearing. The lesions gradually increase in size and number over the entire leaf blade.



FIGURE 3.—Outer leaf of diseased mature cabbage plant showing necrotic rings, vein necrosis, and chlorotic bleaching.

As the leaves become older necrosis appears as spots in the center of the lesion and a little later as concentric rings at the outer margin, giving in the final stage a "bull's-eye" spot in which the dead tissue is brown or blue black (fig. 2). As the disease progresses the tissue

between the initial lesions bleaches and finally becomes dry and brittle (fig. 3). At this stage the leaves drop prematurely, but leaf drop is generally less extensive than in cabbage mosaic (2). Affected leaves occasionally develop asymmetrically. Curling or crinkling of the lamina of the leaf is accentuated only in the later



FIGURE 4.—Stems of young cabbage plants infected with ring necrosis virus showing irregular blue-black lesions on the internodes.

stages of the disease. Necrosis may occur on or along the veins after the necrotic rings in the leaf parenchyma have formed.

Neither Smith (4) nor Tompkins et al. (9) mentioned stem necrosis, which in the present disease commonly occurs in the form of irregular, dark blue to black, slightly sunken areas. The lesions may be small and linear or they may extend irregularly for some distance, usually not involving the leaf scars (fig. 4).

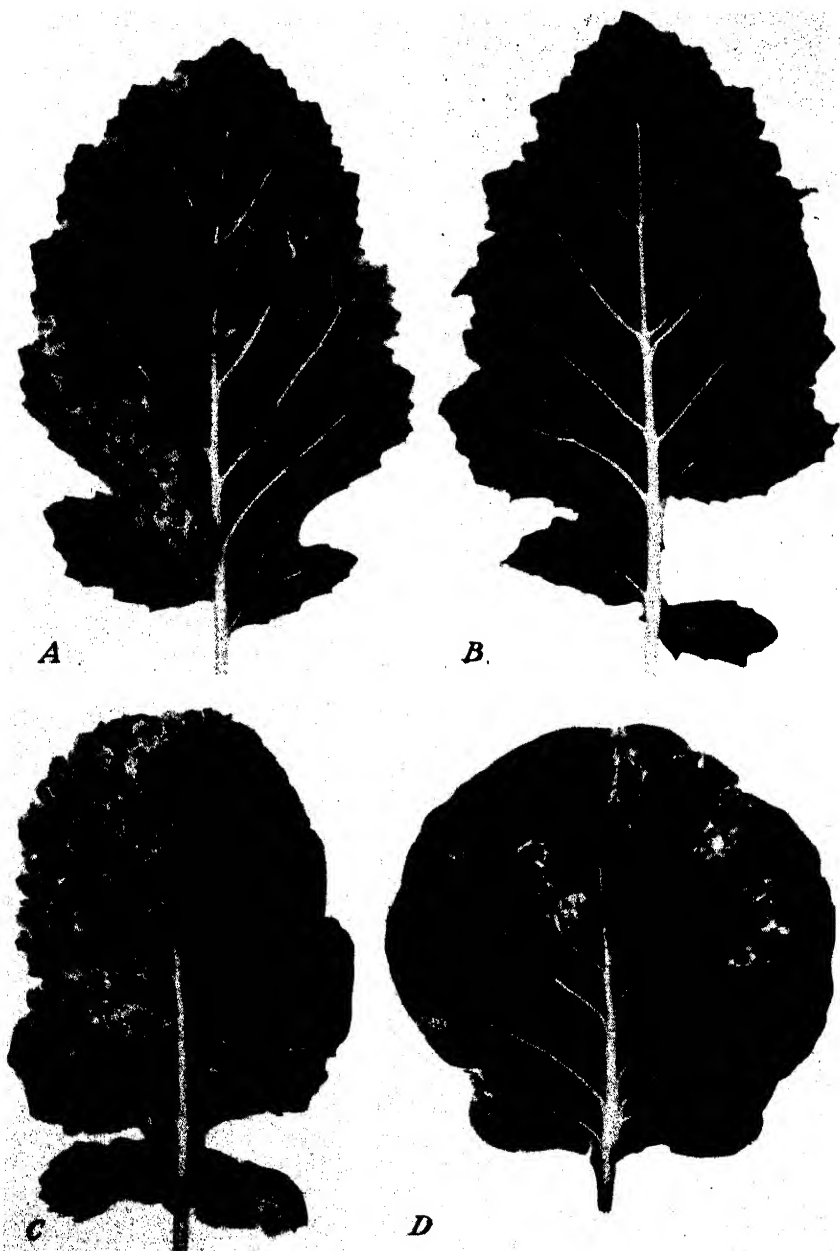


FIGURE 5.—Leaf symptoms of ring necrosis on subspecies of *Brassica oleracea*. A, B, Kohlrabi: A, Diseased; B, uninoculated control; C, Sprouting broccoli; D, brussels sprouts.

Field symptoms vary considerably. Plant growth may be stunted without any sign of chlorosis or necrosis except on the outer head leaves. Outer leaves show varied effects, including chlorosis, unilateral distortion, necrotic rings in interveinal tissue, and linear necrotic lesions on or along the veins. Bleaching of older infected leaves and stem necrosis may occur. Leaf drop is not common as in mosaic, and the internal necrosis of cabbage heads noted in the latter disease (2) has not been observed.

#### OTHER MEMBERS OF BRASSICA OLERACEA

In kale (*Brassica oleracea* var. *viridis*), brussels sprouts (*B. oleracea* var. *gemmifera*), cauliflower (*B. oleracea* var. *botrytis*), sprouting broccoli (*B. oleracea* var. *botrytis*), and kohlrabi (*B. oleracea* var. *gongylodes*), the first symptoms, as in cabbage, appear as small halolike chlorotic lesions, which develop necrotic rings (fig. 5). Conspicuous linear necrotic lesions appear on veins, petioles, and stems.

#### CHINESE CABBAGE

Chinese cabbage (*Brassica pekinensis*) is very susceptible and exhibits somewhat erratic systemic symptoms. The first evidence

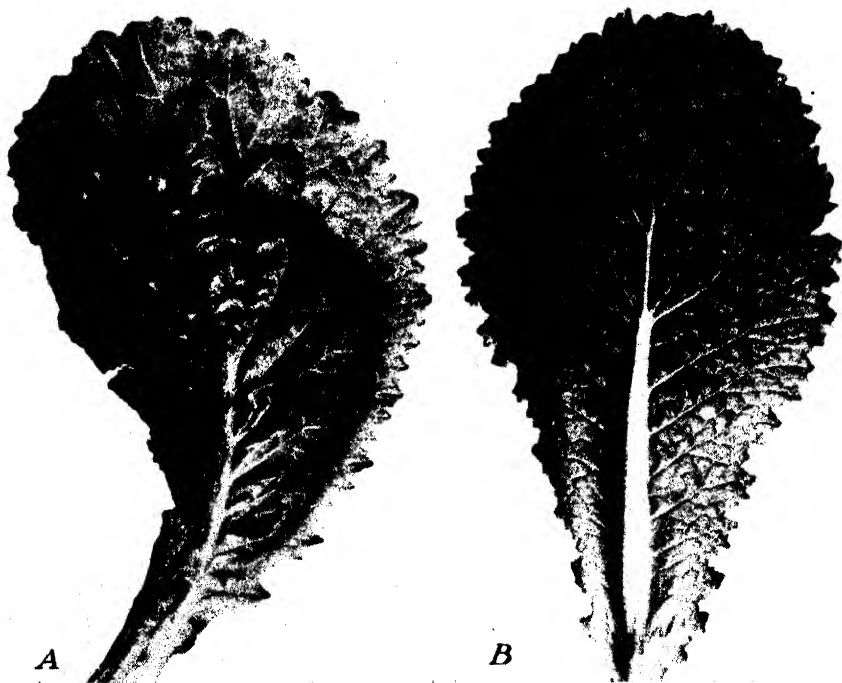


FIGURE 6.—Leaf symptoms produced by systemic infection of Chinese cabbage with ring necrosis virus: A, Leaf distortion, vein necrosis, and necrotic streaking of petiole; B, uninoculated control.

of infection is the appearance of numerous dark specks in the interveinal tissue with slight necrotic streaking on the veins. The lesions may be scattered irregularly but are often confined to one side of the



leaf blade; later when necrotic vein streaking increases and involves the petiole, the symptoms (fig. 6) resemble somewhat those of systemic infection by the black-rot organism, *Bacterium campestre* (Pammel) E. F. Smith (*Phytophthora campestris* (Pammel) Bergey et al.). Well-marked progressive necrotic streaking, severe malformation, and stunting are evident in older infected leaves. The tissues gradually become dry and brittle, and the leaves die prematurely. The virus is occasionally lethal. In contrast to these disease manifestations, Tompkins et al. (9) described only chlorotic rings, some of which became necrotic, on Chinese cabbage infected with black ring.

#### WHITE MUSTARD

Plants of white mustard (*Brassica hirta*) infected with the ring necrosis virus show slight chlorotic lesions on systemically infected leaves, but before ring symptoms fully develop the plants usually die. Tompkins et al. (9) report that black ring disease on this host produces chlorotic rings confined to inoculated leaves with vein clearing on new inner leaves.

#### ANNUAL STOCK

In systemically infected leaves of annual stock (*Mathiola incana* var. *annua*), chlorotic mottling and vein banding develop, followed by bleaching and necrosis. The plants are usually stunted. The flowers show breaking in the form of light flecks or streaks. In black ring on this host (9), mottling and bleaching of the leaves and breaking of flowers occur.

#### DAMES ROCKET AND SIBERIAN WALLFLOWER

In dames rocket (*Hesperis matronalis*) and Siberian wallflower (*Cheiranthus alhionii*), systemic infection results in stunting, conspicuous mottling, and some malformation (fig. 7). The symptoms on these hosts are not unlike those caused by the cabbage mosaic virus (2).

#### RAPE, TURNIP, RUTABAGA, AND RADISH

In rape (*Brassica napus*), turnip (*B. rapa*), rutabaga (*B. campestris* var. *napobrassica*), and radish (*Raphanus sativus*), the early lesions on systemically infected leaves are chlorotic at first, and, as in cabbage, necrotic rings develop later. Various degrees of leaf distortion and stunting occur. It is to be noted that the black ring virus did not infect radish (9).

#### SHEPHERDS-PURSE, GARDEN CRESS, PENNYCRESS, AND HOARY ALYSSUM

Shepherds-purse (*Capsella bursa-pastoris*), garden cress (*Lepidium sativum*), pennycrest (*Thlaspi arvense*), and hoary alyssum (*Berteroa incana*) exhibit slight vein clearing with dwarfing of new growth and chlorosis. In 10 to 15 days after inoculation, early symptoms on older leaves appear as chlorosis of the interveinal areas with a downward curling of the leaf and slight mottling followed by necrosis and defoliation. The younger leaves are malformed and curled, giving the appearance of rosette disease (fig. 8, A).

## NONCRUCIFEROUS HOSTS

Tompkins et al. (9) secured systemic infection with the black ring virus in lambsquarters (*Chenopodium album* L.), sowbane (*C. murale* L.), and spinach (*Spinacia oleracea* L.), although they failed to recover the virus from infected plants. No infection was obtained in sugar beet (*Beta vulgaris* L.) nor Swiss chard (*B. vulgaris* var. *cicla* L.). The writers secured infection in sugar beet, mangel, Swiss chard, and

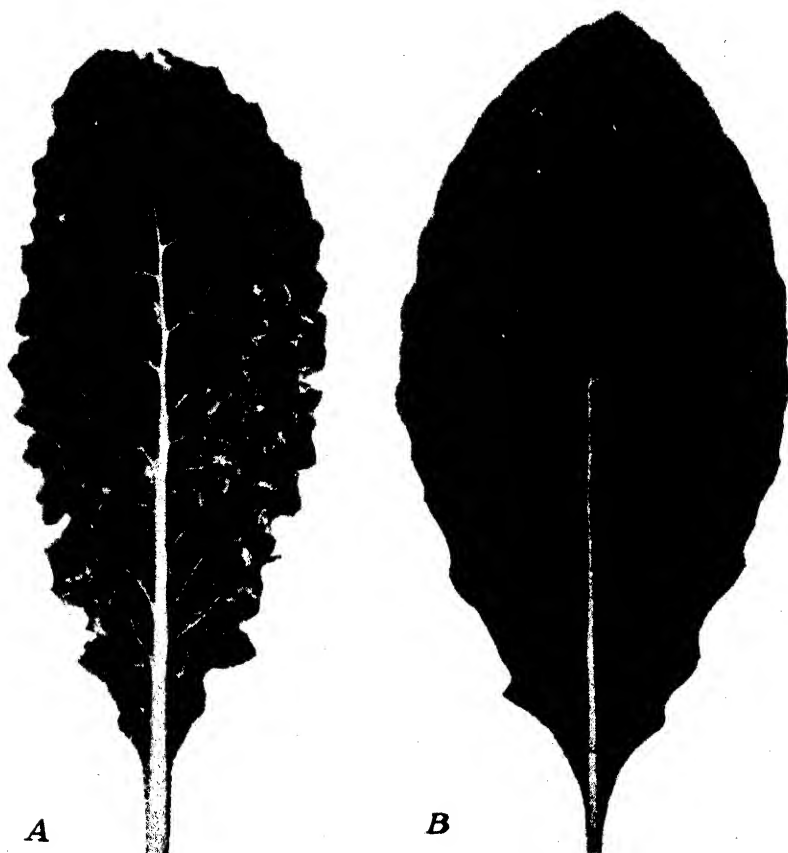


FIGURE 7.—A, Leaf from inoculated dames rocket plant infected with the ring necrosis virus, showing conspicuous mottling; B, uninoculated control.

spinach with the ring necrosis virus. On the first three of these, numerous small dark local spots appeared at the distal portion of the systemically infected leaf without mottling or vein clearing. The spots increased in size and number, and the entire leaf gradually became dry and brittle. Infected spinach leaves (var. Bloomsdale) showed vein clearing and a conspicuous progressively chlorotic mottling. The growing point was stunted and the plant formed a

pathological rosette, while the leaves became distorted and died prematurely (fig. 8, *C*). No infection occurred on lambsquarters.

Most crucifer viruses are infectious to various members of *Nicotiana*. Smith (4) reported that the ring spot virus caused necrotic lesions on inoculated leaves of tobacco (*N. tabacum* L.) in 7 to 10 days. On *N. langsdorffii* Schrank, local lesions and systemic mottling occurred. On *N. glutinosa* L., large local necrotic lesions appeared on inoculated

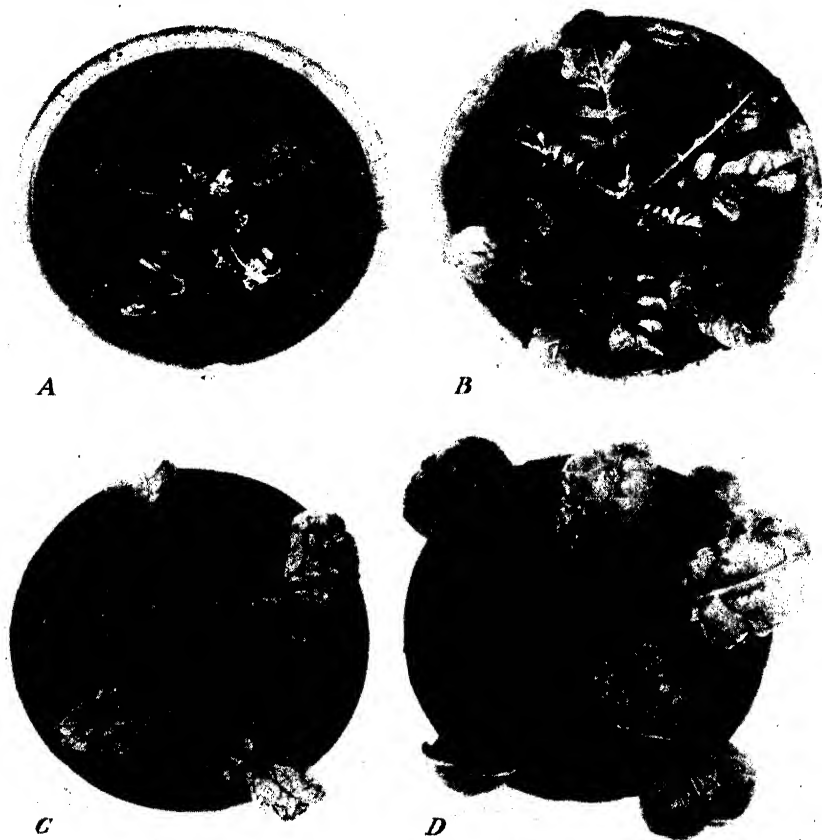


FIGURE 8.—*A, B*, Shepherd's-purse: *A*, Symptoms of systemic infection by ring necrosis virus, showing chlorosis, leaf curl, and dwarfing; *B*, healthy control. *C, D*, Spinach: *C*, Virus-infected plant showing systemic chlorotic mottling and stunting of terminal bud leaves; *D*, healthy control.

leaves, and a mottling of light-green and yellow specks appeared on younger leaves. This disease was commonly fatal to young plants. Rather similar reactions of these three species to the black ring virus are reported (9).

In the case of the ring necrosis virus, necrotic lesions appeared in 3 to 4 days on tobacco (var. Connecticut Havana No. 38). They enlarged rapidly up to 3 mm. or more in diameter, the necrotic tissue forming concentric rings around a reddish center with a darker band

at the margin (fig. 9). No systemic infection occurred. In *Nicotiana glutinosa* no local lesions occurred. The early systemic symptoms are small diffuse chlorotic lesions that become more conspicuous as slight



FIGURE 9.—Necrotic local lesions produced on leaf of *Nicotiana tabacum* by ring necrosis virus.

necrosis develops at the margins. Necrosis gradually involves the entire leaf, but the virus is not fatal to the plant (fig. 10, A). In *N. langsdorffii* as well as in *N. rustica* L. and *N. repanda* Lehm., there

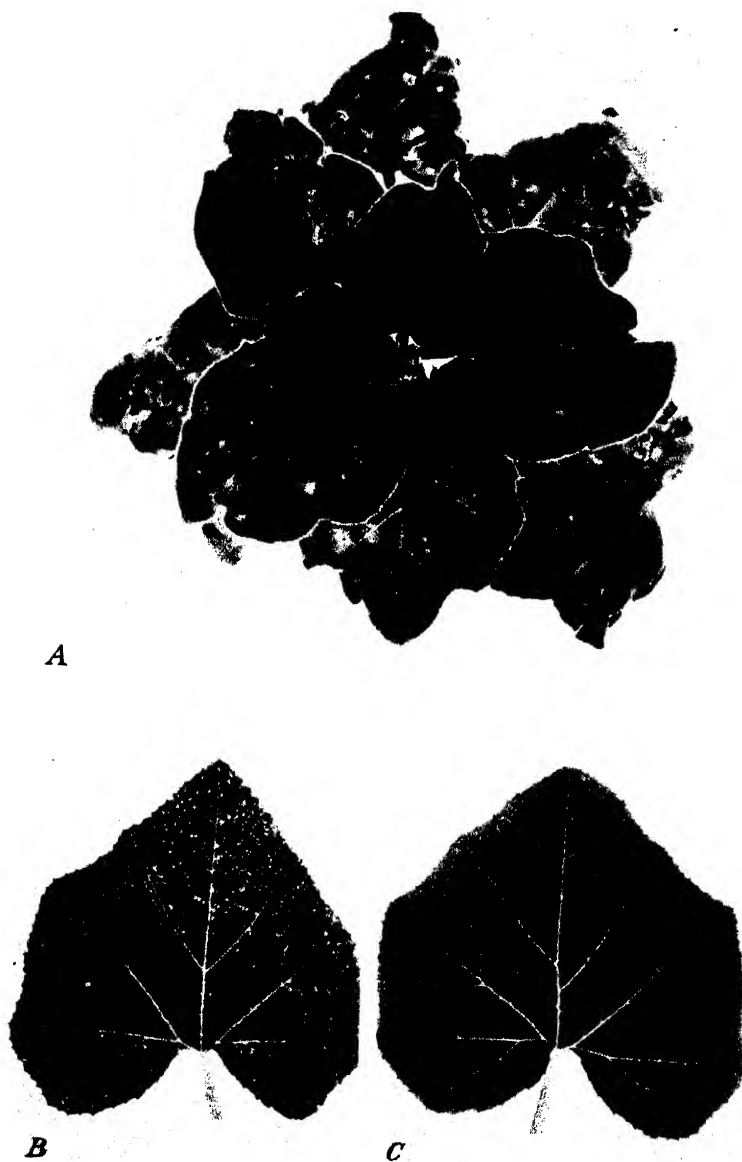


FIGURE 10.—A, Systemic infection of *Nicotiana glutinosa*, showing conspicuous chlorotic lesions on younger leaves and necrotic areas on older infected leaves. B, Numerous small scattered chlorotic lesions on systemically infected leaf of cucumber; no symptoms appear on the inoculated leaves. C, Leaf from healthy cucumber plant (uninoculated control).

are likewise no local lesions. The systemic symptoms, like those of the cabbage mosaic virus (2), are chlorosis and pronounced mottling (fig. 11).

In petunia ( $\times$  *Petunia hybrida* Vilm.) the black ring virus caused chlorotic rings, curling and puckering of leaves, and stunting. Infection of this plant (var. Summer Pink) with ring necrosis virus is characterized by slight vein clearing and small irregular scattered chlorotic lesions with little or no necrosis. Bleaching and dropping of leaves follow, and there is considerable stunting. Sectorial breaking

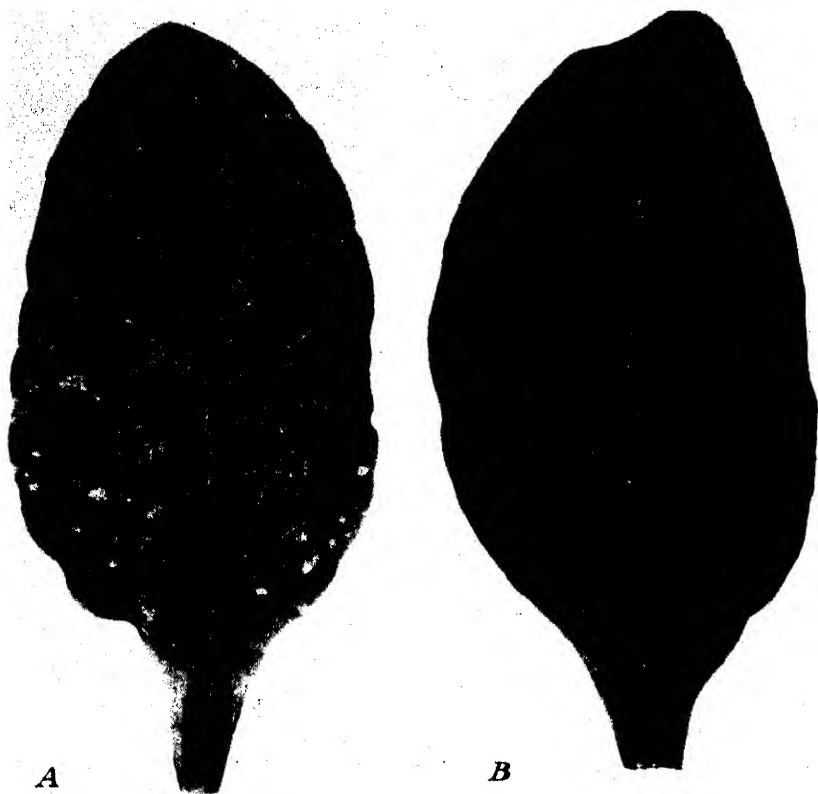


FIGURE 11.—Symptoms produced on leaves of *Nicotiana glauca* by systemic infection with ring necrosis virus: A, Systemically infected leaf showing conspicuous, irregular chlorotic lesions; B, leaf from uninoculated control.

in the form of streaks results in malformed and undersized flowers (fig. 12, A and C).

*Zinnia* (*Zinnia elegans* Jacq., var. Fantasy Yellow) when infected shows vein clearing and slight mottling followed by leaf twisting and severe stunting. In calendula (*Calendula officinalis* L., var. Ball's Orange) the first symptoms appear as vein clearing and are followed by chlorosis and stunting.

The black ring virus did not infect cucumber (*Cucumis sativus* L.). When the ring necrosis virus was inoculated in the Chicago Pickling

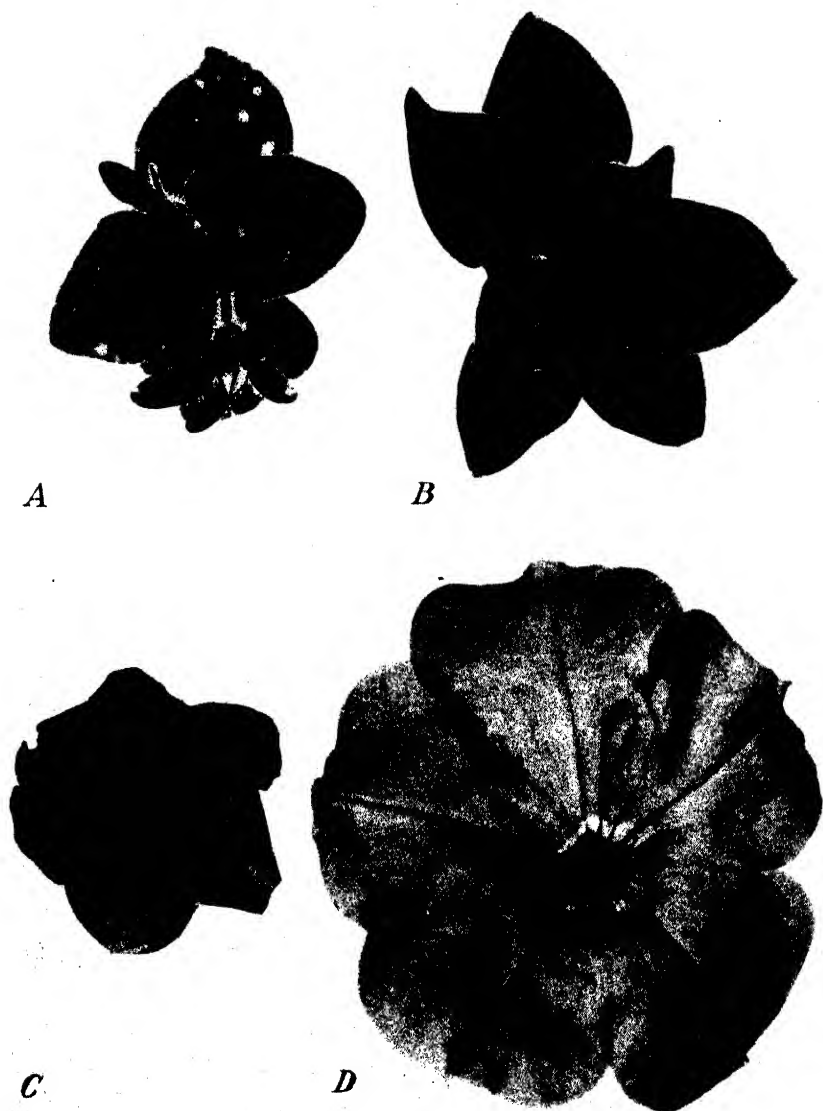


FIGURE 12.—Symptoms produced by ring necrosis virus on petunia: *A*, Vein clearing and scattered chlorotic lesions of leaves on normal green background; *B*, uninoculated control; *C*, sectorial breaking of flower, accompanied by distortion and stunting; *D*, uninoculated control.

variety systemic symptoms appeared as numerous small chlorotic lesions (fig. 10, B).

Infection was not obtained on the following species in attempts to inoculate them by the abrasive method or by the transfer of aphids known to be vectors of the ring necrosis virus: Lambsquarters (*Chenopodium album* L.); broadbean (*Vicia faba* L.); nasturtium (*Tropaeolum majus* L.), var. Golden Gleam; pansy (*Viola tricolor* L.), var. Black King; celery (*Apium graveolens* L.), var. Golden Self-Blanching; tomato (*Lycopersicon esculentum* Mill.), var. Globe; currant tomato (*L. pimpinellifolium* (Jusl.) Mill.); potato (*Solanum tuberosum* L.), var. Irish Cobbler and Rural New Yorker; nightshade (*S. nigrum* L.); eggplant (*S. melongena* L.), var. Black Beauty; *Nicotiana sanderae* Bailey; *N. longiflora* Cav.; *N. nudicaulis* S. Wats.; jimsonweed (*Datura stramonium* L.); snapdragon (*Antirrhinum majus* L.), var. (rust-resistant strain); muskmelon (*Cucumis melo* L.), var. Milwaukee Market; watermelon (*Citrullus vulgaris* Schrad.), var. Stone Mountain; dandelion (*Taraxacum officinale* Weber); China-aster (*Callistephus chinensis* Nees), var. Giant Blue (wilt-resistant strain); French marigold (*Tagetes patula* L.), var. Harmony; and florists cineraria (*Senecio cruentus* (L'Hér.) DC.), var. Multiflora Nana. Subsequent reinoculations to young cabbage and tobacco with the extracted juice from the foregoing inoculated plants failed to cause infection.

#### TRANSMISSION OF THE VIRUS

The strain of the ring necrosis virus used in the experimental studies on insect transmission was obtained from systemically infected mature cabbage grown at Madison, Wis. After the establishment of the virus on healthy cabbage seedlings by mechanical inoculation, a constant source of fresh inoculum was maintained in the greenhouse on this host. The virus remained virtually unchanged through successive transfers. All inoculations were conducted in a greenhouse where the temperature usually ranged from 22° to 25° C. and where weekly fumigation was practiced for the control of insects. The artificial inoculations of test plants were regularly made by dusting the leaves with powdered carborundum and lightly rubbing with a small piece of absorbent cotton soaked with juice extracted from diseased cabbage plants.

Studies on insect transmission of this virus involved the use of the green peach aphid (*Myzus persicae* (Sulz.)) and the cabbage aphid (*Brevicoryne brassicae* (L.)). The methods of culture and transfer of the aphids were essentially the same as those described in a recent paper (2). Healthy cabbage seedlings infested with nonviruliferous aphids and uninoculated plants free of aphids served as controls. Reinoculations from all test plants for the recovery of the virus were made to cabbage seedlings to determine the presence of the ring necrosis virus. It was determined by repeated trials that both species of aphid transmit the ring necrosis virus readily.

#### PROPERTIES OF THE VIRUS

Undiluted juice expressed from recently infected cabbage was used for the study of the properties of the ring necrosis virus. Young cabbage plants were used as test plants. The results are given in table 1.



TABLE 1.—*Properties of ring necrosis virus in vitro as determined by infection on cabbage*

[25 plants used in each test]

Longevity in vitro				Thermal inactivation				Tolerance to dilution			
Period aged at 20°-22° C. (hours)	Plants infected in trial No.—			Temperature (10-minute period)	Plants infected in trial No.—			Dilution	Plants infected in trial No.—		
	1	2	3		1	2	3		1	2	3
	No.	No.	No.	°C.	No.	No.	No.		No.	No.	No.
0	25	25	25	40	25	25	25	0	25	23	24
6	25	25	25	43	24	22	23	1:25	22	23	24
12	24	23	22	45	22	21	20	1:50	16	15	14
24	18	16	20	48	14	15	13	1:100	6	5	7
36	3	2	4	50	0	0	0	1:500	2	2	2
48	0	0	0	52	0	0	0	1:600	0	0	0
60	0	0	0	54	0	0	0	1:700	0	0	0

In table 2 the inactivation points recorded in the literature for other crucifer viruses are listed in comparison with those of the ring necrosis virus. Insofar as these physical properties are concerned, the latter resembles most closely the cabbage black ring virus and the cabbage mosaic virus, but differs from them very definitely in having lower thermal and dilution inactivation points. It has many points of difference in host range and symptoms from all others except the cabbage black ring virus. As already stated, in symptoms produced on cabbage it resembles the black ring and ring spot viruses. The last of these is not described adequately enough by Smith (4) to permit comparison, because no physical properties were recorded and the host range and symptoms were studied in only a limited way. The chief distinctions between the ring necrosis virus and the black ring virus, aside from the differences in physical properties just mentioned, are infectivity of radish, sugar beet, and chard by the former, the difference in optimal temperatures for disease development, and differences in symptoms on various common hosts already mentioned.

TABLE 2.—*Inactivation points of ring necrosis virus for longevity in vitro and for resistance to high temperatures and dilutions, compared with those recorded for other crucifer viruses*

Virus	Authority	Point at which inactivation occurred after indicated treatment		
		Period aged in vitro	Temperature (10-minute period)	Dilution
		Hours	°C.	
Ring necrosis	Larson and Walker	48	50	1:600
Turnip mosaic	Hoggan and Johnson (1)	72	54	1:100,000
Cauliflower mosaic	Tompkins (5)	360	75	1:2,000
Chinese cabbage mosaic	Tompkins and Thomas (10)	96	75	1:6,000
Turnip mosaic	Tompkins (6)	48	63	1:4,000
Cabbage black ring	Tompkins et al. (9)	48	57	1:1,000
Mild stock mosaic	Tompkins (7)	144	60	1:5,000
Severe stock mosaic	do	192	60	1:4,000
Radish mosaic	Tompkins (8)	384	68	1:15,000
Cabbage mosaic	Larson and Walker (2)	72	55	1:2,000

## SUMMARY

Ring necrosis is a virus disease of cabbage, which is here described in comparison with other crucifer virus diseases.

The virus infects all crucifers tested, including some 25 species and subspecies or botanical varieties, and certain other noncruciferous hosts, including sugar beet, chard, spinach, tobacco, *Nicotiana glutinosa*, *N. langsdorffi*, *N. rustica*, *N. repanda*, petunia, zinnia, and calendula.

The virus is transmitted readily by mechanical inoculation and by the green peach and cabbage aphids.

The physical properties investigated are distinct from those already reported for other crucifer viruses.

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# MERCURIC CHLORIDE AS A PRESERVATIVE OF CYANOGENETIC PLANTS FOR CHEMICAL ANALYSIS<sup>1</sup>

By REINHOLD R. BRIESE, *assistant chemist*, and JAMES F. COUCH, *formerly chemist, Pathological Division, Bureau of Animal Industry, United States Department of Agriculture*

## INTRODUCTION

In a previous publication<sup>2</sup> it was shown that cyanogenetic plants could be preserved for chemical analysis by means of mercuric chloride for periods ranging from 3 months for dried plants to 6 months for fresh plants. Attention was called to the fact that plants so preserved gave higher yields of hydrocyanic acid on analysis than plants not so preserved, indicating that the customary methods of analysis lead to low results in most cases with considerable error in a number of instances.

The study was continued to determine how long samples of cyanogenetic plants might be preserved with different quantities of mercuric chloride without undergoing appreciable loss of hydrocyanic acid. Since mercuric chloride exerts an inhibiting effect on the enzyme, the study was designed also to determine the length of time the enzyme requires to liberate the maximum obtainable amount of hydrocyanic acid with the different concentrations of mercuric chloride used.

## EXPERIMENTAL PROCEDURE

The general procedure was the same as that outlined in the earlier publication.<sup>3</sup>

Fourteen varieties of sorghum grown at the United States Bureau of Plant Industry experimental farm at Arlington, Va., were placed at the disposal of the authors through the courtesy of John H. Martin of that Bureau. During the years 1937 and 1938, 159 collections of material from these varieties were made. The tissues were comminuted either by slicing young plants finely or by mincing older plants in a food chopper. The product was immediately mixed thoroughly and samples, usually 50 gm., of the mixed mass were weighed for the various treatments as rapidly as possible to minimize the loss of hydrocyanic acid which occurs when ground green plant is exposed to the air. In order to obtain uniformity at the beginning of the experiment, in a series of samples that were to be compared, all the samples were covered with water at the same time. The hydrocyanic acid content in one sample was then determined in water by immediate distillation without maceration and in another after maceration for 24 hours. The remainder of the samples were weighed into pint fruit jars, which were then filled with water containing, in solution, 1 to 5 percent of mercuric chloride, calculated on weight of plant. The preserved samples were analyzed at intervals of several weeks to 2 years. The number of samples preserved from each collection depended on the

<sup>1</sup> Received for publication October 17, 1940.

<sup>2</sup> BRIESE, REINHOLD R., and COUCH, JAMES F. PRESERVATION OF CYANOGENETIC PLANTS FOR CHEMICAL ANALYSIS. *Jour. Agr. Res.* 57: 81-107, illus. 1938.

<sup>3</sup> BRIESE, REINHOLD R., and COUCH, JAMES F. See footnote 2.

quantity of material and time available and on the object in view. A moisture determination was made on each collection of plant. Care was exercised to see that the volume of water used in the preservations was uniform and not large. It has been found that diluting the preservative with water during maceration diminishes its preserving power. When mercuric chloride was added in proportion to the increased volume of water added, the preserving power was maintained, but a longer time was required for complete cyanogenesis in the sample.

Samples of two species of wild cherry (*Prunus melanocarpa* and *P. serotina*) and one species of arrowgrass (*Triglochin maritima*) were also obtained in 1937 and 1938 and preserved in 1 to 5 percent of mercuric chloride.

The samples of *Prunus melanocarpa* and of *Triglochin maritima* were collected, ground, and preserved by E. A. Moran of the Bureau of Animal Industry at the experiment station at Salina, Utah. The material was preserved immediately after collection and no moisture determinations were made. The samples were then shipped to the laboratory in Washington, D. C., where the analyses were made. Under these conditions it was not possible to carry out the analyses in water by immediate distillation or after 24-hour maceration.

The *Prunus serotina* was collected in Arlington, Va., immediately before use. The leaves were ground in a food chopper, mixed, and rapidly weighed, usually 50-gm. samples being taken. At this point the series of samples was quickly flooded with water to assure the greatest possible uniformity in the series, as in the sorghums. The hydrocyanic acid content of one sample was then determined in water by immediate distillation without maceration and in another after maceration for 24 hours. The remainder of the samples were preserved in 1 to 5 percent of mercuric chloride calculated on weight of plant and analyzed at suitable intervals. A moisture determination was made on each collection of *P. serotina*.

#### SOURCES OF ERROR

The greatest difficulty in preservation was encountered with young plants, owing to the fact that cyanogenesis is more rapid in young tissue, and loss of hydrocyanic acid while the samples are being prepared for preservation is unavoidable. This loss would be less in routine analysis because fewer samples would be used than in the present experiment and less time, therefore, would elapse between mincing or slicing of the plants and immersion in the preservative. This fact accounts in part for the 9 out of a total of 145 analyses in which the values for the unpreserved samples were higher than or equal to those for samples preserved in mercuric chloride. In the early part of the season of 1937, before the main sources of error were recognized, the samples for analysis without preservation were weighed first and sometimes immersed in water before the remainder of the samples were weighed for preservation. In order that the sample for immediate analysis might be distilled by noon and the sample for 24-hour maceration might be ready for distillation the next day early enough to be finished by noon, these two samples were immersed in water as soon as they had both been weighed. Meanwhile, weighing of the series was suspended for a few minutes.

Distillation was begun immediately on the first sample. The samples for preservation were then rapidly weighed, but the short time that had elapsed was no doubt sufficient to allow a greater loss of hydrocyanic acid than in the case of the two samples not preserved. This source of error was eliminated as soon as the 4-week preserved samples were analyzed and the error discovered. Even with this advantage, more than 93 percent of the unpreserved samples gave lower results than were obtained after preservation. In some cases the former were 20 to 30 percent lower than the latter.

In comparing the samples for the efficiency of the preservative in different strengths and for different periods of time, 3 percent was allowed for experimental error, this allowance being based on previous findings. Differences greater than 3 percent were considered as significant.

### EFFICIENCY OF 1 PERCENT OF MERCURIC CHLORIDE

In the experiments involving 1 percent of mercuric chloride, the results obtained with sorghums are presented in tables 1 and 2, and those with wild cherry and arrowgrass in table 3.

TABLE 1.—*Hydrocyanic acid obtained from green sorghum in 1937 after maceration in water only and in water containing 1 percent of mercuric chloride calculated on weight of plant*

Collection No.	Variety	Height of plant	Moisture content	Hydrocyanic acid per 100 gm. of dry plant obtained after —							
				No maceration	Maceration for 24 hours in water	Maceration with 1 percent of mercuric chloride in solution for the designated number of weeks					
						4	12	26	52	78	104
		Inches	Percent	Mg.	Mg.	Mg.	Mg.	Mg.	Mg.	Mg.	Mg.
99	Ajax	60-72	68.62		16	16					
17	Chiltex	8-10	80.73	41	46						
15	Dwarf yellow milo	12-14	81.11	21	43						
16	do	10-13	83.75	81	127	132					
26	do	30	79.08		53	67	75	69	68		76
43	do	8-10	87.14		221	215	218	215			206
22	Hegari	18	85.42	62	99	108					
30	do	30-36	79.63	25	41	47			44		
57	do	36-48	76.86	15	35			35			
60	do	20-22	80.40	140	225	238					
62	do	36	75.54	129	149	244		250	242		226
72	do	60-72	76.93	81	122	189	182	188	182	191	174
81	do	60	73.14	45	107	124		135			
83	do	6-12	88.91	243	330	341	356	344			
84	do	60-72	70.46	67	133	146					
89	do	60-72	74.75	56	126	147					
96	do	60-72	70.82	33	83	93		88			66
102	do	60-72	70.79	24	55	63		63			
103	do	60-72	74.19	33	55	68		77			
104	do	72-84	76.23	21	42	50	47	48			
105	do	72	73.05	17	28	31					
107	do	72	72.22	15		45		44			
112	do	72-84	62.76	10	21	28					
116	do	72-84	70.70	13	23	29		24			
19	Kansas Orange sorgo	16-18	86.71	55	92	111		110	110		
20	do	6-8	83.03	97	119	122					
42	do	6-8	85.11		234	263			263		
32	Sharon kafir	3-6	83.49	92	104	98	111	107	104		101
46	do	10-12	85.58	101	126	139					
49	do	10-12	86.23	53	104	116			112		
52	do	14-16	87.14	82	96	138	140				
55	do	20-22	84.03	57	76	96					
59	do	30-36	86.42	119	174	182					
63	do	36		32	57	65					

TABLE 1.—*Hydrocyanic acid obtained from green sorghum in 1937 after maceration in water only and in water containing 1 percent of mercuric chloride calculated on weight of plant—Continued*

Collection No.	Variety	Height of plant	Moisture content	Hydrocyanic acid per 100 gm. of dry plant obtained after —							
				No maceration	Maceration for 24 hours in water	Maceration with 1 percent of mercuric chloride in solution for the designated number of weeks					
						4	12	26	52	78	104
		Inches	Percent	Mg.	Mg.	Mg.	Mg.	Mg.	Mg.	Mg.	Mg.
68.	Sharon kafir	36	77. 12	34	69	79	75	82			
74.	do	60	82. 23	32	61	70	65	63			
77.	do	60-72	75. 55	29	58	73		68			
87.	do	60-72	74. 15	13	24	31	28	27			
94.	do	60-72	74. 52	11	9	20		15			
101.	do	72	76. 10	12	20	21		18			
111.	do	72	74. 78	8	10	11					
115.	do	72	74. 06	12	13	16					
120.	do	72	72. 95	8	8	15					
14.	Spur foterita	3- 6	82. 81	278	207	277					
27.	do	3- 5	82. 95	263	303	271					
34.	do	24-30	82. 23	50	89	96	96				
44.	do	3- 6	85. 87			290			292		
45.	do	8	85. 45	213	253	265			275		
47.	do	24-30	78. 46	24	46	52			47		
48.	do	8-10	86. 15	183	190	233	226	240	238		
50.	do	30-36	76. 27	22	41	47			48		
51.	do	14-16	87. 29	205	241	260					
53.	do	20-24	80. 72	127	176	214	215				
54.	do	20-24	86. 80	128	180	188	203				
58.	do	36-48	85. 20	76	91	102		91			
64.	do	48-60	76. 71	114	162	202		204			180
66.	do	6-12	85. 69	191	220	270		270	264		256
67.	do	6-12	83. 18.	137	193	228					
69.	do	72	75. 63	82	154	172	167				
73.	do	72	73. 71	68	121	163	151	151		125	
76.	do	72-84	73. 17	36	73	81		77			
78.	do	12-20	85. 37	121	174	174		178			
80.	do	72-84	72. 19	43	86	96	100	96			
82.	do	12	87. 78	237	269	301	303	318			
86.	do	84-96	72. 61	36	76	83	84	83			
88.	do	84-96	66. 24	21	43	49		46			
93.	do	84-96	71. 36	31	36	41	41	37			
100.	do	84-96	70. 25	10	16	16		16			
106.	do	60-72	70. 50	20	34	48					
110.	do	84-96	64. 69	21	38	49		50			
113.	do	12	85. 41	164	191	228	235	234		226	
114.	do	84-96	70. 67	12	14	21		19			
117.	do	6	84. 12	227	243	300					
119.	do	84	72. 22	8	8	21					
23.	Sumac sorgo	14	86. 61	31	53	66		67	61		66
24.	do	20	88. 93	54	72	106		106	101		104

† Second-growth plants; all others first growth.

TABLE 2.—*Hydrocyanic acid obtained from green sorghum in 1938 after maceration in water only, and in water containing 1 percent of mercuric chloride (calculated on weight of plant), except as otherwise indicated in footnotes*

Collection No.	Variety	Height of plant	Moisture content	Hydrocyanic acid per 100 gm. of dry plant obtained after—							
				No maceration	Maceration for 24 hours in water	Maceration with mercuric chloride in solution for the designated number of weeks					
						4	8	26	52		
		Inches	Percent	Mg.	Mg.	Mg.	Mg.	Mg.	Mg.		
63.	Ajax	36	74.38	65	103	117					
92	do	72	72.95	28	57	71	71				
108	do	60-72	72.42	19	29	38	38				
105	Atlas sorgho	96-108	74.50	12	24	28	28				
60	Dwarf Yellow milo	60	77.94	44	68	83	84				
93	do	84-96	71.51	16	25	40	40				
76	Grohoma	72-84	73.67	28	63	69	72				
91	do	72-84	73.73	23	49	56	69				
106	do <sup>1</sup>	10-12	85.67	96	128	139	138				
124	do <sup>2</sup>	6-10	82.63	70	132	158	138	155			
31	Hegari	6	82.51		236	288		325			
33	do	6-8	84.36		324		296	337	329		
40	do	8-12	89.47		409		312	316			
43	do	15-22	81.24		171		209	267			
45	do	30-36	83.16		176		218				
47	do	30	79.08		93		135	157			
48	do	30	67.55		113		121				
49	do	30	75.02		109		114				
50	do	30	75.91		95		134				
51	do	30	70.55	60			99				
52	do	30	79.02	89		115	117				
53	do	30	78.28	70		129	142				
54	do	30	71.34		82		99				
55	do	30	69.94	43	78		89				
56	do	30	75.73	46	91		113				
58	do	36	82.44	134	127	173	193				
61	do	36	81.96	105		88	73				
62	do	36	81.55	152		159	193				
64	do	42-48	77.09	121	157	175	266				
65	do	36	80.73	148	138	142					
70	do	60	81.68	134	140	180	218				
71	do <sup>1</sup>	6-12	89.08	217	276	289	300				
73	do	48	82.40	77	144	128	151				
74	do	36-48	81.80	77	99	132	145				
79	do	60-72	72.74	78	89	145	154	159	157		
87	do	72-84	76.62	68	127	136	141				
95	do <sup>2</sup>	6-10	87.91	280	288	323	367	385	379		
97	do	72-84	74.92	59	129	140					
102	do	72-84	78.10	40	70	88	82				
107	do	60-72	83.64	95	193	208	210				
110	do	72-84	74.46	28	58	72	69	66			
112	do <sup>2</sup>	6-10	88.20	214	385	439	482				
113	do	72-84	72.71	15	26	32	31				
118	do	72-84	73.25	47	86	99	104				
127	do <sup>2</sup>	6-8	85.55	216	263	329	397		105		
84	Kansas Orange sorgho	84-96	75.61	46	78	93	101				
98	do	96	75.82	19	54	57	63		66		
123	do <sup>2</sup>	10-12	86.62	37	53	98	98		106		
81	Leoti sorgho	96	72.55	24	56	50	60				
96	do	96	74.60	13	27	35	35				
83	Rex	96	75.84	33	48	65	68				
99	do	96	68.75	13	21	29	26				
78	Sagrain	72-84									
94	do	72-84	75.06	15	42	43	43				
116	do <sup>1</sup>	6-10	87.01	95	140	155	154				
34	Sharon kafir	8-12	85.26		145		168		175		
46	do	36	83.91		75		88		108		
67	do	48-60	78.30	24	43	48	50				
75	do	72	76.51	19	46	41	41				
82	do	72	76.49	23	33	34	38				
90	do <sup>2</sup>	6-12	85.50	79	89	104	106	109	110		
103	do	60-72	75.05	16	25	29	29				
115	do <sup>2</sup>	12-14	86.41	35	36	93	97	94	85		
125	do	72	72.11	10	14	13	16	22			

<sup>1</sup> 2 percent of mercuric chloride.<sup>2</sup> Second-growth plants; all others first growth.



TABLE 2.—*Hydrocyanic acid obtained from green sorghum in 1938 after maceration in water only, and in water containing 1 percent of mercuric chloride (calculated on weight of plant) except as otherwise indicated in footnotes—Continued*

Collection No.	Variety	Height of plant	Moisture content	Hydrocyanic acid per 100 gm. of dry plant obtained after—							
				No maceration	Maceration for 24 hours in water	Maceration with mercuric chloride in solution for the designated number of weeks				Mg.	Mg.
						4	8	26	52		
		Inches	Percent	Mg.	Mg.	Mg.	Mg.	Mg.	Mg.		
57	Spur feterita	36-48	80.16	108	145	163	171				
69	do	72	74.84	110	166	187	188				
86	do	72-84	75.22	74	122	139	140		198	183	
88	do <sup>2</sup>	6-12	86.83	202	211	260	270				
104	do	6-10	87.44	363	388	463	484		478	485	
111	do <sup>2</sup>	8-12	88.22	262	444	447	436		464	413	
119	do <sup>2</sup>	8-14	81.33	46	164	186	190		198	183	
122	do <sup>2</sup>	8-10	85.13	105	227	256	275				
126	do <sup>2</sup>	10-12	85.03	78	154	174	208		190		
128	do <sup>2</sup>	6-7	84.49	146	155	236					
35	Sumac sorgo	8-10	85.16		233		246			257	
42	do	12-16	85.65		205		272				
59	do	60	80.81	83	114	158					
66	do	72-84	79.04	62	85	120	130	131			
77	do	84-96	72.56	46	95	114	114				
80	do	84-96	73.10	51	100	107	107				
89	do	84-96	76.71	51	90	108	110				
114	do	96	75.23	30	58	76	78	71			
							84				
120	do <sup>2</sup>	6-10	87.21	103	176	220	232	258			

<sup>1</sup> 2 percent of mercuric chloride.<sup>2</sup> Second-growth plants; all others first growth.TABLE 3.—*Hydrocyanic acid obtained from wild cherry (Prunus melanocarpa and P. serotina) and from arrowgrass (Triglochin maritima) after maceration in water only or in water containing 1 percent of mercuric chloride calculated on weight of plant*

Collection No.	Plant	Moisture content	Hydrocyanic acid per 100 gm. of plant macerated in --							
			Water for 24 hours	Mercuric chloride for the designated number of weeks						
				4	8	13	26	52	78	104
		Percent	Mg.	Mg.	Mg.	Mg.	Mg.	Mg.	Mg.	Mg.
37-69	<i>Prunus melanocarpa</i> <sup>1</sup>			139	136	136	126	118	115	105
37-69A	do <sup>1</sup>			126					126	
37-70	do <sup>1</sup>			120	122	121	115	112		
5-37	<i>P. serotina</i> <sup>2</sup>	73.22	428				462	426		
9-37	do <sup>3</sup>	65.93	281		244	232	249			
11-37	do <sup>3</sup>	65.14	255	252		256	251	238		
33-37	do <sup>3</sup>	62.64	438	457			460			
37-61	<i>Triglochin maritima</i> <sup>1</sup>			101				98	89	
37-62	do <sup>1</sup>			122	131	130	129	121	114	104
37-62A	do <sup>1</sup>			59			59			
37-68	do <sup>1</sup>			12		13	10	10		

<sup>1</sup> Calculated to green plant for which moisture determinations are not available.<sup>2</sup> 30 weeks.<sup>3</sup> Calculated to moisture-free plant.<sup>4</sup> 35 weeks.

In the case of the sorghums, for each period of time in both years, a count was made of the samples with upward, stationary, and downward trends in hydrocyanic acid content. The data are given in table 4. In determining the trend in a series of samples, it was necessary to ignore certain obvious errors. For example, if the 8-week sample

was lower than the 4-week one, and the 26-week sample was more than 3 percent higher than the 4-week sample, then the trend of the 26-week sample was upward and therefore the trend of the 8-week sample was considered to be upward also.

TABLE 4.—Percentage of total number of sorghum samples, after indicated number of weeks in 1-percent mercuric chloride in solution, showing trends in hydrocyanic acid content

Trend	Percentage of total samples after—							
	8 weeks (1938)	12 weeks (1937)	26 weeks (1937)	26 weeks (1938)	52 weeks (1937)	52 weeks (1938)	78 weeks (1937)	104 weeks (1937)
	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>
Upward	55	43	15	47	6	0	0	0
No change	39	44	51	29	47	38	33	30
Downward	6	13	34	24	47	62	67	70
Total samples	<i>Number</i> 51	<i>Number</i> 23	<i>Number</i> 39	<i>Number</i> 17	<i>Number</i> 17	<i>Number</i> 8	<i>Number</i> 3	<i>Number</i> 10

As mentioned previously, more than 93 percent of the unpreserved samples gave lower results than were obtained after preservation. Table 4 shows that of the samples analyzed in 1938 after 8 weeks of preservation, 94 percent were higher than or equal to the 4-week samples in hydrocyanic acid content, no 12-week samples having been considered. Of those analyzed in 1937 after 12 weeks of preservation, 87 percent were higher than or equal to the 4-week samples, no 8-week samples having been considered. For both years 70 percent of the samples preserved for 26 weeks gave figures higher than or equal to those for 8 or 12 weeks. Of the 26-week group, the samples having a downward trend were 17 in number, 4 of which were near the limit of experimental error, 5 were from 6 to 10 percent low, and 8 were from 10 to 20 percent low.

The time required for complete cyanogenesis, as judged from table 4, varied considerably. For the intervals of 8, 12, 26, and 52 weeks the percentages of samples showing an upward trend were 55, 43, 25, and 4, respectively. Thus with 1 percent of mercuric chloride, 25 percent of the samples required between 8 and 26 weeks for complete cyanogenesis.

In the case of wild cherry and arrowgrass (table 3), with the use of 1 percent of mercuric chloride there was good preservation for 8 to 13 weeks and in most instances for 26 weeks. However, it appears that 1 percent is not sufficient to preserve material of these species when the content of hydrocyanic acid is large.

#### EFFECT OF HIGHER CONCENTRATIONS OF MERCURIC CHLORIDE

Data obtained after the preservation of samples in concentrations of mercuric chloride of 1 to 5 percent and for periods of 4 to 78 weeks, (except for one instance of 104 weeks) are presented for sorghum in table 5 and for wild cherry and arrowgrass in table 6. The higher concentrations of mercuric chloride appeared to give better preservation in some cases but necessitated a longer period for complete cyanogenesis because of the increased inhibition of the enzyme with increasing concentration of mercuric chloride.

TABLE 5.—*Hydrocyanic acid obtained from sorghums after maceration with various quantities of mercuric chloride in solution for different intervals of time*

Collection No.	Variety	Mois- ture con- tent	Time of macera- tion	Hydrocyanic acid per 100 gm. of dry plant obtained after maceration in mercuric chlo- ride solution of the designated strengths in percent <sup>1</sup>				
				1	2	3	4	5
		Percent	Weeks	Mg.	Mg.	Mg.	Mg.	Mg.
45-38	Hegari	83. 16	8	145	218	168	131	95
			4	154	138	124	128	118
79-38	do.	72. 74	8	151	153	153	154	144
			12	159	154	159	159	144
			26	157	159	157	160	157
			52	161	161	162	165	166
			78	323				
95-38	do. <sup>2</sup>	87. 91	4	367	342			
			8	385	389			
			26	379				
			52	390				
			78	99				
118-38	do.	73. 25	4	104	100			
			8	105				
			26	68	69		69	70
			4	69	68		68	70
			13	69				
			15	69				
36-130	do. <sup>3</sup>	14. 02	17	68	68			
			19	65	70		69	
			21	63	69		70	70
			30		68		69	69
			39					69
			52				68	69
115-38	Sharon kafir	86. 41	4	93				
			8	97	103			
			26	94	110			
			52	85	91			
104-38	Spur feterita <sup>2</sup>	87. 44	4	463				
			8	484	465			
			13	496	500			
			26	478	479			
			52	485	482			
111-38	do. <sup>3</sup>	88. 22	4	447				
			8	436	420			
			26	464	442			
			52	413				
119-38	do. <sup>2</sup>	81. 33	4	186				
			8	190	191			
			12	202				
			26	198	199			
			52	183	210			

<sup>1</sup> Calculated on weight of plant.<sup>2</sup> Second growths; all others first growth.<sup>3</sup> Dried plant.

TABLE 6.—*Hydrocyanic acid obtained from wild cherry (Prunus melanocarpa and P. serotina) and from arrowgrass (Triglochin maritima) after maceration in various quantities of mercuric chloride in solution for different intervals of time*

Collection No.	Plant	Moisture content	Time of preservation	Hydrocyanic acid per 100 gm. of dry plant obtained after maceration in—					
				Water	Mercuric chloride solution of the designated strengths in percent <sup>1</sup>				
					1	2	3	4	5
		Percent	Weeks	Mg.	Mg.	Mg.	Mg.	Mg.	Mg.
38-91	<i>P. melanocarpa</i>	74.98	8		825				
			26		833	805			
			52		754	794			
38-92	do	77.66	8		895				
			26		888	905			
			52		811	863			
38-152	do	63.35	8	251	313	309	301	284	293
			13	239	314	311	306	301	318
			26	201	316	311	327	323	327
38-173	do	61.11	52		291	312	318	306	314
			8	302	347	353		347	338
			26	232	348	373	374	359	363
108-37	<i>P. serotina</i>	67.01	8		339	337			
			13		346	345			
			26		346	343			
21-38	do	68.05	4			309	308	308	
			12			313	313	304	
			26		299	315	313	316	304
117-38	do	57.75	52		292	321	325	319	305
			104			319		306	
			4		512	535			
38-95	<i>Triglochin maritima</i> <sup>4</sup>		8		515	546			
			12			555			
			26		505	544	531		543
			52		471	503			
			78			543			
			8	136	178	166			
			13	143	174	166	160	147	141
			26	132	171	167	159	158	149
			78		155	160	160	155	150

<sup>1</sup> Calculated on weight of plant.

<sup>2</sup> 1 percent of mercuric chloride added 2 months after beginning of experiment.

<sup>3</sup> 1.5 percent of mercuric chloride.

<sup>4</sup> Calculated to green plant.

For sorghum, tables 2 and 5 show that in 12 of 14 cases the 2-percent 26-week samples were higher than the 2-percent 8-week samples. Therefore, in many cases, for 2-percent mercuric chloride more than 8 weeks are required for complete cyanogenesis in sorghum. Of the 26-week samples in 2-percent mercuric chloride, all were higher than or equal to the 1-percent 8-week samples of the same collections. The three comparable 12- and 13-week samples (table 5) showed that in these three cases cyanogenesis was completed in 3 months. In only one case did a 2-percent 26-week sample show a loss as compared with a shorter period. In only one case did a 2-percent 26-week sample with upward trend show a lower value than the 1-percent 26-week sample with upward trend. It seems safe to conclude that, for sorghum in 2-percent mercuric chloride, cyanogenesis will be complete in 3 or 4 months and that the liberated hydrocyanic acid will be preserved in almost all cases for 6 months.

The increased efficiency of 2 percent as compared with 1 percent of mercuric chloride in the preservation of wild cherry (*Prunus serotina*) is seen by comparing the data in tables 3 and 7. In table 7 no losses, except such as were within the limits of experimental error, were

evident in samples preserved in 2 percent of mercuric chloride for 6 months and in the sample preserved for 1 year. With 1 percent of mercuric chloride (table 3), there was good preservation for 8 to 13 weeks and in most cases for 6 months.

TABLE 7.—*Hydrocyanic acid obtained from fresh wild cherry (Prunus serotina) after maceration in water only or in water containing 2 percent of mercuric chloride calculated on weight of plant*

Collection No.	Hydrocyanic acid per 100 gm. of dry plant after maceration in —						
	Water for—		2-percent mercuric chloride solution for—				
	0 hours	24 hours	4 weeks	8 weeks	13 weeks	26 weeks	52 weeks
	Milligrams	Milligrams	Milligrams	Milligrams	Milligrams	Milligrams	Milligrams
20-38	338		378				
21-38	247		300				
30-38	648	646	731		313		321
32-38	472		553	563		315	
37-38	356	350	400			410	
117-38	332	454	535	546	555	544	

In the case of green sorghum (second sample in table 5), 2 percent of mercuric chloride preserved as well as 3 percent for the period covered (78 weeks). For dried sorghum (fifth sample in table 5) 3 percent preserved better than 2 percent for a period of more than 20 weeks.

From the data in table 6 it seems safe to conclude that, for wild cherry, in most cases 2 percent of mercuric chloride is as satisfactory a preservative as 3 percent, especially for intervals up to 26 weeks. In the third sample of wild cherry 3 percent of mercuric chloride preserved somewhat better than 2 percent and as well as 4 or 5 percent.

For both sorghum and wild cherry higher concentrations of mercuric chloride than 3 percent do not offer advantages to offset the increased inhibition of cyanogenesis.

In the sample of green *Triglochin maritima* used, however, the highest figures were obtained with 1 percent of mercuric chloride, indicating that the enzyme of this plant is sensitive to the preservative. One percent of mercuric chloride is sufficient to combine with the total hydrocyanic acid found in the sample, since 1 gm. of the salt will combine with 199.2 mg. of the acid. However, many other substances in green plants will combine with mercuric chloride so that an excess of the salt should be employed.

#### RAPIDITY OF FORMATION OF HYDROCYANIC ACID IN PRESERVED SAMPLES

Since it was known that mercuric chloride inhibits cyanogenesis in plants, it was of interest to determine how rapidly the hydrocyanic acid is formed, particularly during the first 4 weeks of preservation. Data for the rate at 2-hour intervals during the first 24 hours of preservation for wild cherry (*Prunus melanocarpa*), arrowgrass, sorghum, and Johnson grass have already been published,<sup>4</sup> together with a few data for longer intervals up to 13 days. Data obtained at weekly intervals up to 4 weeks, and for longer intervals thereafter,

<sup>4</sup> BRIESE, REINHOLD R., and COUCH, JAMES F. See footnote 2.

with the use of 1 percent of mercuric chloride, are presented in table 8. It was found that in these samples the maximum figure, within experimental error, developed in 3 to 4 weeks with two exceptions, in which the maximum developed at 5 to 13 weeks. Data for 8-week samples are not available, but table 4 shows that for the 1938 sorghums 55 percent of the 8-week samples gave more hydrocyanic acid than the 4-week samples. Table 8 shows that cyanogenesis in the presence of mercuric chloride is rapid for wild cherry but is slow with sorghum. This finding is consistent with other evidence, which indicates that the enzyme in wild cherry is very much more active than in sorghum. The highest yield of hydrocyanic acid that can be obtained by maceration in water is ordinarily obtained after 2 to 3 hours with wild cherry but only after 24 to 48 hours with sorghum.

TABLE 8.—*Rapidity of formation of hydrocyanic acid in green cyanogenetic plants macerated in water containing 1 percent of mercuric chloride calculated on weight of plant*

Collection No.	Plant	Moisture	Hydrocyanic acid per 100 gm. of dry plant obtained after maceration in—									
			Water for 24 hours	1-percent mercuric chloride solution for the designated number of weeks								
				1	2	3	4	13	26	52	104	
		Percent	Mg.	Mg.	Mg.	Mg.	Mg.	Mg.	Mg.	Mg.	Mg.	
6-37	<i>Prunus serotina</i>	65.60	309	313	312	312	327	---	---	---	---	
8-37	do	62.44	<sup>2</sup> 125	121	123	123	123	---	122	---	---	303
10-37	do	69.08	<sup>2</sup> 303	312	312	312	295	<sup>3</sup> 321	<sup>4</sup> 304	---	---	
13-37	Spur feterita	85.80	73	77	81	80	82	88	89	82	---	82
18-37	Sorghum var. Chiltex	83.90	91	95	102	104	101	---	---	---	---	
21-37	Kansas Orange sorgo	87.72	125	118	126	---	128	---	129	121	---	
31-37	Spur feterita	82.25	179	155	157	177	175	175	---	<sup>5</sup> 180	---	161

<sup>1</sup> 65 weeks.

<sup>2</sup> Not macerated, the macerated sample being lower.

<sup>3</sup> 5 weeks; same figure for 6 weeks.

<sup>4</sup> 30 weeks.

<sup>5</sup> 78 weeks.

#### ATTEMPTS TO INCREASE THE RATE OF CYANOGENESIS IN PRESENCE OF MERCURIC CHLORIDE

A number of experiments were made to determine whether a technique might be developed by means of which the time for maximum development of hydrocyanic acid in the presence of mercuric chloride could be shortened. In one experiment 50 gm. of fresh second-growth Spur feterita was heated for 7 hours under a reflux condenser in a mixture of 1,500 cc. of water and 0.5 gm. of mercuric chloride. On the following day the hydrocyanic acid was determined as 139 mg. per 100 gm. calculated to dry plant. The quantity obtained after maceration of this sample for 4 weeks in 1 percent of mercuric chloride was 236 mg. Evidently the refluxing was not effective in causing complete hydrolysis of the glucoside. Other attempts were made in which the quantity of preservative was varied and maceration stopped after 1 to 4 days with and without the addition of an enzyme preparation made from sorghum and known to be active. The results are presented in table 9. A comparison of the quantity of hydrocyanic acid obtained after such treatment with that determined

by 4 to 8 weeks' maceration of the same sample in 1 percent of mercuric chloride shows that, in 13 experiments, the figure obtained by the former method was higher in 1 instance and the same, within experimental error, in 2 instances as with the latter method. In 10 instances results with the latter method were higher, the difference being more than 100 percent in sample No. 64-38 of hegari. In two cases the addition of enzyme made little difference, but with sample No. 63-38 of Ajax, there was a loss in the hydrocyanic acid obtained when enzyme was added.

TABLE 9.—Comparison of quantity of hydrocyanic acid obtained after adding enzyme to fresh sorghum macerated in mercuric chloride solution, with that obtained from a similar sample macerated for 4 to 8 weeks in water containing 1 percent of mercuric chloride, calculated on weight of plant

Collection No.	Variety	Enzyme added	Period of maceration	Mercuric chloride added	Hydrocyanic acid per 100 gm. of dry plant obtained after—	
					Treatment	Maceration for 4 to 8 weeks in 1 percent of mercuric chloride
			Days	Percent	Mg.	Mg.
63-38	Ajax	Before maceration	4	0.5	68	117
63-38	do	None	4	.5	90	117
45-38	Hegari	do	1	.5	141	218
58-38	do	do	2	.5	190	193
58-38	do	After maceration	2	.5	208	193
58-38	do	Before maceration	2	.5	197	193
64-38	do	None	2	.5	92	206
27-38	Scarborough broomcorn	do	1	.5	75	153
46-38	Sharon kafir	Before maceration	1	.5	82	108
69-38	Spur feterita	do	3	1.0	177	198
69-38	do	do	7	1.0	183	198
59-38	Sumac sorgho	do	2	.5	124	156
59-38	do	None	2	.5	126	156

#### STABILITY OF MERCURIC CYANIDE SOLUTIONS

The apparent loss of hydrocyanic acid in the above-cited case of Ajax sorghum raised the question whether mercuric cyanide is stable in the presence of cyanogenetic enzymes. It is known that free hydrocyanic acid is slowly converted into other substances by these enzymes<sup>6</sup> and if the same process occurred with mercuric cyanide it would influence the preservative efficiency of mercuric chloride with cyanogenetic plants.

A solution of mercuric cyanide in water was adjusted to contain 21.2 mg. of hydrocyanic acid per 25 cc. To 1 liter of this solution was added 13.3 gm. of an active dhurrinase preparation from sorghum; to 1 liter, 13.3 gm. of an active prunase preparation from *Prunus melanocarpa*. A third liter was kept under the same conditions as a control. The solutions were kept in the dark at 25° C. in pyrex flasks with rubber stoppers. At intervals 25-cc. portions were withdrawn and the hydrocyanic acid content was determined. The mercuric

<sup>6</sup> COUCH, JAMES F., and BRIESE, REINHOLD, R. THE DESTRUCTION OF HYDROCYANIC ACID BY PRUNASE AND THE INFLUENCE OF SUGARS ON THE REACTION. Wash Acad. Sci. Jour. 29: 219-221, illus. 1939.

cyanide was decomposed with stannous chloride and the freed acid was distilled into 5-percent sodium hydroxide, the distillate being then titrated according to the Liebig-Denigès method. The results are presented in table 10. At the end of 15½ weeks each solution had lost 2.8 percent of hydrocyanic acid. It was evident that the cyanogenetic enzymes did not hasten decomposition of mercuric cyanide under the conditions of this experiment.

TABLE 10.—*Stability of mercuric cyanide solutions at 25° C. in the presence of cyanogenetic enzymes*

Time of storage (weeks)	Hydrocyanic acid recovered per 25 cc. of solution			Time of storage (weeks)	Hydrocyanic acid recovered per 25 cc. of solution		
	Control	With dhurrinase	With prunase		Control	With dhurrinase	With prunase
	<i>Milligrams</i>	<i>Milligrams</i>	<i>Milligrams</i>		<i>Milligrams</i>	<i>Milligrams</i>	<i>Milligrams</i>
0.....	1 21.2	1 21.2	3 21.1	5.....	20.7	20.7	20.7
1.....	21.1	20.9	20.5	6.....	20.8	20.5	20.7
2.....	21.2	20.9	20.7	10.....	20.9	20.7	20.6
3.....	20.8	20.9	20.8	15½.....	20.6	20.6	20.5
4.....	20.8	20.8	20.7				

<sup>1</sup> pH 6.10, at end 6.50.

<sup>2</sup> pH 6.67, at end 5.79.

<sup>3</sup> pH 6.32, at end 5.19.

## OTHER MERCURY COMPOUNDS AS PRESERVATIVES

One set of experiments was made to determine the comparative efficiency of mercuric chloride, mercuric oxide, and mercurous chloride as preservatives. A collection of fresh hegari leaves was preserved in the usual manner with each of these compounds in a concentration of 1 percent based on the green weight of the plant. The samples were analyzed after 1, 4, and 6 months. The results are presented in table 11. Neither of the other two compounds tested was so efficient as mercuric chloride. After 1 month the mercuric oxide sample had lost 20 percent and after 4 months the mercurous chloride sample had lost over 15 percent. At the end of 6 months these samples had lost still more, whereas the mercuric chloride sample had lost none. In all three samples the quantity of mercury present was much in excess of that needed to combine with all the hydrocyanic acid present.

TABLE 11.—*Hydrocyanic acid recovered from fresh hegari macerated for various periods with different mercury compounds*

Period of preservation (months)	Hydrocyanic acid per 100 gm. of dry plant after maceration with 1 percent of—		
	Mercuric chloride	Mercurous chloride	Mercuric oxide
	<i>Milligrams</i>	<i>Milligrams</i>	<i>Milligrams</i>
1.....	35	35	28
4.....	36	29	26
6.....	35	26	24



# INFLUENCE OF HYDROGEN-ION CONCENTRATION ON PRESERVATION

Cyanogenetic plants develop their maximum content of hydrocyanic acid under certain optimum conditions of acidity.<sup>6</sup> It was therefore of interest to determine the influence of acidity on the development of the hydrocyanic acid during preservation. Samples preserved in the usual way were used; results are presented in table 12. It was found that the increased acidity due to the mercuric chloride was beneficial, the average pH of the samples falling within the optimum range. No evidence was obtained that hydrochloric acid derived from the mercuric chloride was formed in excess or repressed cyanogenesis in the mixtures.

TABLE 12.—*Hydrocyanic acid content and pH of samples of hegari macerated with and without the use of mercuric chloride*

Collection No.	Mercuric chloride used <sup>1</sup>	Time of maceration	pH of sample			Hydrocyanic acid recovered per 100 gm. of plant
			At beginning	At end	Average	
	Percent	Days				Milligrams
29-38.....	0	1	5.63	4.74	5.19	171
62-38.....	0	1	5.86	4.19	5.03	123
62-38.....	1	28	5.28	5.34	5.31	159
62-38.....	2	56	5.05	4.14	4.6	193
95-38 <sup>2</sup> .....	0	1	5.47	3.92	4.7	288
95-38.....	1	56	5.19	4.23	4.71	367
95-38.....	2	56	5.07	3.99	4.53	342

<sup>1</sup> Calculated on weight of plant.

<sup>2</sup> Second-growth, all others first growth.

## SUMMARY AND CONCLUSIONS

A further study was made of the mercuric chloride method for preserving cyanogenetic plants for chemical analysis. Experiments were made with 14 varieties of sorghum, 2 species of wild cherry, and 1 species of arrowgrass. This study extends and confirms the results previously reported.

On the whole, good preservation was obtained for periods up to 6 months and, in a number of cases, for 1, 1½, and 2 years. Young plants are more difficult to preserve than older plants. This is due to their higher content of hydrocyanic acid, to the rapidity with which it is developed in bruised tissues, and also to the fact that the cyanogenetic enzyme appears to be much more susceptible to the inhibiting action of mercuric chloride than is the case in older tissues.

A concentration of 1 percent of mercuric chloride based on the weight of the plant sample is sufficient to preserve specimens that contain 100 to 125 mg. of hydrocyanic acid per 100 gm. Specimens that contain larger quantities of the acid require 2 to 3 percent of mercuric chloride for satisfactory preservation. Concentrations larger than 3 percent offer no advantages but have the disadvantage of markedly inhibiting cyanogenesis.

The rate of cyanogenesis in the plants studied when they were preserved in 1 percent of mercuric chloride is such that in general the

<sup>6</sup> COUCH, JAMES F., and BRIESE, REINHOLD R. HYDROGEN ION CONCENTRATION AND CYANOGENESIS IN SORGHUM. Amer. Jour. Pharm. 111: 55-64, 151-160, 193-201. 1939.

maximum amount of hydrocyanic acid is liberated in 3 to 8 weeks. With higher concentrations of the mercury compound the maximum quantity may not be attained for 6 to 12 months.

The rate of cyanogenesis in the preserved samples could not be regularly increased by the addition of the appropriate enzyme to the sample.

The cyanogenetic enzymes dhurrinase and prunase did not affect the stability of a solution of mercuric cyanide over a period of 15½ weeks.

Mercuric oxide and mercurous chloride were less efficient as preservatives than mercuric chloride.

The hydrogen-ion concentration of mixtures of sorghum and mercuric chloride falls within the optimum pH range for cyanogenesis in sorghum. The addition of mercuric chloride may be regarded as beneficial from this standpoint.

Figures for the hydrocyanic acid obtained from samples of plants macerated in water, except for a few irregular cases, fall much below those obtained by the use of mercuric chloride. Water maceration cannot be relied upon to furnish accurate data for the hydrocyanic acid content of cyanogenetic plants.



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## GLYCOGEN IN *PRODENIA ERIDANIA*, WITH SPECIAL REFERENCE TO THE INGESTION OF GLUCOSE<sup>1</sup>

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### INTRODUCTION

The importance of carbohydrates as a reserve food material for various animal organisms is well known. A reserve food supply is particularly important to insects such as the southern armyworm (*Prodenia eridania* (Cram.)) because of its relatively long period of pupation. Large numbers of the southern armyworm have been reared in the laboratories of the Division of Control Investigations for use in various physiological experiments. The insects have been reared in greenhouse cages and the larvae fed turnip and collard foliage, a diet high in carbohydrates. An investigation of some phases of carbohydrate metabolism in this insect has therefore been undertaken. The glycogen content has been determined at daily intervals throughout the life cycle, and the effect of the ingestion of glucose on the blood-glucose level and the tissue-glycogen content of sixth instars has been studied. The results of these experiments are reported in this paper following a review of the findings of other investigators with regard to the carbohydrate metabolism of various insects.

### REVIEW OF LITERATURE ON CARBOHYDRATE METABOLISM IN INSECTS

#### OCCURRENCE OF GLYCOGEN

Glycogen was first demonstrated in insects by Bernard (8, pp. 113-114),<sup>2</sup> who described the larvae of "asticots" as "veritable sacks of glycogen." Bernard also found glycogen in the pupae of the same insects. Külz (60) found glycogen in the larvae, but not in the eggs, of *Calliphora vomitoria* (L.) (*Muscida vomitoria*).<sup>3</sup> Tichomiroff (86) showed that the yolks of hibernating eggs of silkworms (*Bombyx mori* L.) contained glycogen, but that during development the glycogen disappeared. Anderlini (3) found glycogen in all stages of the silkworm, the largest quantities occurring in the female pupae. In a preliminary report Bataillon and Couvreur (6), although giving no analytical data, found glycogen to be at a maximum the day after nymphosis, and then to decline rapidly until the amount remaining

<sup>1</sup> Received for publication August 31, 1940.

<sup>2</sup> Italic numbers in parentheses refer to Literature Cited, p. 525.

<sup>3</sup> Scientific names of insects in parentheses immediately following other scientific names are those appearing in the reference cited. Other scientific names are those currently in use according to the Division of Insect Identification of the Bureau of Entomology and Plant Quarantine, U. S. Department of Agriculture.

was too small to estimate accurately. Bataillon (5) later extended these observations and included his data. Dubois and Couvreur (31) found that glycogen reached a maximum of 53 mg. per six insects on the sixth day after climbing. The amount remaining at the end of the pupal period was insignificant.

Vaney and Maignon (91, 92, 93), reporting the daily changes that occur during the metamorphosis of the silkworm, found the greatest amount of glycogen (1.59 percent) just at the formation of the chrysalis, then a decline until emergence, and another rise after copulation. By histological methods they demonstrated glycogen in the cells of the fat body, in leucocytes, and in muscles, but not in the silk glands or the digestive epithelial or hypodermic cells. Eggs within the female moths contained glycogen. Tichomirow's (86) finding that glycogen diminished during the development of the egg was confirmed. The eggs contained 2.85 percent of glycogen 2 to 4 days after oviposition and only 0.87 percent 7 to 8 days thereafter. In both pupae and adults the female contained considerably more glycogen than the male, the greatest difference being in the adults.

Bogojavlensky (18) found glycogen especially concentrated in the external lobes of the fat body of the silkworm, and present to a lesser extent in the internal lobes. Glycogen was also found in other organs, but Bogojavlensky concluded that this glycogen was slightly different from the fat-body glycogen since it seemed to have a different solubility. He found considerable glycogen in the midgut and in the terminal part of the rear gut. The test for glycogen in the silk glands was doubtful, although Bogojavlensky considered its presence probable. Glycogen depots were relatively abundant in the conjunctive tissue of the envelopes of the genital organs and were also present in the tracheae. Glycogen was always present in the walls of dorsal vessels and in pericardial cells, also in oenocytes and ganglia of the abdominal nervous system. It was often absent in muscle.

Using the roach *Blatta orientalis* L. (*Stylopyga orientalis*), Philipstschenko (73) found that glycogen was abundant in the cells of the larval fat body but was scarce in those of the adults. No seasonal variation in the amount of glycogen present in the tissues of the honeybee (*Apis mellifera* L.) was noted by Parhon (70), although she found a considerable seasonal variation in combustion and respiratory exchange.

Kotake and Sera (58) found a steady decrease in glycogen during the metamorphosis of the silkworm. As the exact age of their insects was not stated, they may have started their analyses too late to note the rise reported by other investigators.

According to Ronzoni and Bishop (77), carbohydrates found in the honeybee may be designated (1) as free sugar, determined as glucose, which is found in the blood and possibly to some extent in the cells; (2) as combined carbohydrate, part of which is a polysaccharide of lower molecular weight than glycogen and part of which is combined with protein in the blood; and (3) as glycogen. Glycogen is laid down exclusively in the cells but is released into the blood during the late stages of histological break-down.

The amount of carbohydrate present after acid hydrolysis was determined by Inouye (50) for various stages of the silkworm. Larvae, pupae, and adults contained 4.98, 4.37, and 5.71 percent, respec-

tively, calculated on a dry basis. Yamakawa (97) confirmed the results of other investigators in finding a decrease of glycogen during metamorphosis. Eggs of a single-brood strain of silkworms contained the largest amount of glycogen soon after they were laid, and the glycogen content then decreased steadily, according to Vaney and Conte (90). Straus (82) found that glycogen first appeared in the honeybee the third day in its larval life, and increased rapidly to a peak at pupation. At this time workers contained 32.48 percent of glycogen and drones 25.52 percent. The glycogen declined steadily until it had almost disappeared at emergence, but it rose again in the adult. According to Sturtevant (83), bees dead from foulbrood contained almost no glycogen. Pigorini (75) confirmed the results of previous workers as to the changes that occur in the glycogen content of the silkworm egg during development.

Ditman and Weiland (28) found that the metabolism of glycogen in pupae of the corn earworm, *Heliothis armigera* (Hbn.) (*Heliothis obsoleta* (F.)), was largely dependent on the temperature. At 86° F. values for the accumulation and decrease in the quantity of glycogen present were similar to those reported for other insects.

The development of *Gasterophilus intestinalis* (Deg.) (*Gastrophilus equi*) was studied over an 8-month period by Kemnitz (56, 57), who found that between 31.1 and 14.1 percent of the dry weight was glycogen. He also found larvae of an unknown species of *Chironomus* to contain 16.67 percent of glycogen. Shinoda (81) found that pupae of the wild silk moth (*Dietyoploca japonica* (Moore)) contained only small quantities of glycogen, fat being its main reserve material.

The blowfly (unnamed species), according to Frew (37), did not contain glycogen or any other carbohydrate that yielded glucose on acid hydrolysis; both larvae and pupae were examined. Rudolfs (79) analyzed the tent caterpillar (*Malacosoma americana* (F.)) for glycogen throughout its life cycle. The glycogen fell from 2.79 percent at hatching to a few tenths of 1 percent when fully grown. In the same time the glycogen actually present increased from 27.9 mg. per 100 larvae to 375 mg. Rudolfs found glycogen in the muscles, leucocytes, fat glands, and eggs.

In *Culex pipiens* L., De Boissezon (19) found that glycogen was concentrated in certain fat cells, or trophocytes, of both larvae and imagoes. Because he did not find glycogen in the intestinal cells during digestion, he thinks that glycogenesis takes place in the fat cells.

Blanchard and Dinulescu, in an analysis of larvae (15) and pupae (16) of *Gasterophilus intestinalis*, *G. nasalis* (L.), and *G. haemorrhoidalis* (L.), found that glycogen rose steadily during the larval stage and fell off sharply during inanition. Most of their data were repeated by Dinulescu (27).

Evans (33) reported finding glycogen in the blowfly *Lucilia sericata* (Meig.), but from his description of the material he isolated this finding must be questioned. The material Evans isolated differed from glycogen in that it gave a reddish-brown color with iodine which did not return after boiling and no reduction was obtained after boiling with dilute acid. Total carbohydrate in the insect was obtained by hydrolyzing dried insect material with acid, clarifying, and determining reducing sugars by the Hagedorn-Jensen method.

Heller (44) found that the pupae of *Deilephila euphorbiae* (L.) contained 0.78 percent of glycogen. Timon-David and Gouzon (89) found that *Geonica utricularia* (Pass.) (*Pemphigus utricularius*) and *Pemphiga cornicularia* (Pass.) contained 2.007 percent of glycogen. Glycogen was not found in significant amounts in either the pupae or the prepupae of the wax moth (*Galleria mellonella* (L.)) by Crescitelli and Taylor (24). Beutler (10) did not find in the body of the honey-bee any reserve carbohydrates from which the insect could replenish its blood sugar during starvation. Bialaszewicz (12) found that in the silkworm glycogen rose abruptly from 6 to 31.6 mg. on the sixth day after the fourth molt, dropped to 21 mg. during spinning, and rose again to 45 mg. in the pupal stage. Roy (78) could not detect glycogen in the body fluid of the wax moth. Babers (4) found 3.29 mg. of glycogen per 100 cc. of blood in the hemolymph of the sixth-instar southern armyworm. Paillot (69), using histological methods, found an increase in glycogen during the metamorphosis of the silkworm, the largest amounts being present in the fat tissue and epithelial cells of the digestive tube. He then stated that glycogen was not found in blood cells, pericardial cells, oenocytes, silk glands, Malpighian tubes, and probably not in muscle, but elsewhere in the same article he reported the presence of glycogen in oenocytes, pericardial cells, and occasionally in the Malpighian tubes. Kuwana (61) found 50 mg. of glycogen per 100 cc. of larval blood of the silkworm. During pupation glycogen rose to 130 mg. per 100 cc., the females consistently having more than the males.

#### SOURCE OF GLYCOGEN

Protein as a source of glycogen was suggested by Külz (60). He found that larvae of *Calliphora vomitoria* (*Muscida vomitoria*) that had been reared from glycogen-free eggs on egg white and horse meat contained glycogen, and he concluded that protein was the source of this glycogen. His conclusion does not necessarily hold true, however, since both egg white and horse meat contain carbohydrates. Although he did not mention glycogen by name, Abderhalden (1) found that the beetle *Anthrenus museorum* (L.) could synthesize all needed foodstuffs, both carbohydrates and fats, as well as chitin, from silk threads which were free from both fat and carbohydrate.

Couvreur (23) suggested that fat was the source of glycogen in the silkworm, since he obtained a rise in the glycogen content that corresponded to a fall in the fat content. Dubois and Couvreur (31) also considered fat to be a source of glycogen. Bataillon (5) considered the origin of glycogen to be histolytical, but Vaney and Maignon (91) did not think this probable, for they found glycogen in the largest quantities in cells that were most active during metamorphosis. Vaney and Maignon maintained that the fat-body cells resembled the liver cells of vertebrates in acting as centers for the elaboration of fat and glycogen. This view was also taken by Bogojavlensky (18), who found reserve glycogen mainly in the fat body of silkworms. He believed that it was formed locally from the monosaccharides obtained from the food. Weinland (95), from his work on *Calliphora*, did not consider the evidence presented by Couvreur sufficient to warrant the assertion that fat was the source of glycogen. Kotake and Sera (58) also disagreed with Couvreur; they found that both glycogen and fat decreased steadily during the chrysalis stage, but Yamakawa (97)

thought that in this stage carbohydrate was formed from fat and then utilized by the silkworm.

Kemnitz (57) presented evidence that glycogen in *Gasterophilus intestinalis* (*Gastrophilus equi*) was formed from the proteins in the stomach of the horse and later transformed into fat. The work of Kaneko (52) indicated that glycogen was built up in the gut epithelium of the silkworm from the carbohydrates in mulberry leaves. Bitō (14) found that silkworm pupae from larvae that had fed on leaves of *Cudrania triloba* contained less fat and glycogen than pupae from larvae fed on mulberry leaves, apparently because of the higher protein content of the *Cudrania*. Kato (53) found that, when partly mature mulberry leaves were fed to silkworm larvae, the digestion of soluble carbohydrates and the accumulation of glycogen in both larvae and pupae were greater than when older and younger leaves were fed. The addition of cane sugar in moderate amounts to the normal diet of the silkworm increased the weight of the body, cocoon, and silk. Kato, Miwa, and Negi (54) also found that the general health of the insects was improved, but no criterion of health was given. They found that 90 percent of the added sugar was digested, resulting in a remarkable increase in the fat and glycogen content of the insect. Kuwana (61) found that, after ingestion of glucose by silkworm larvae, the amounts of reducing substances in the blood following acid hydrolysis were greatly increased. He thought part of this was due to glycogen and perhaps part to a glucoprotein complex.

#### CHITIN

The metabolism of chitin has attracted the attention of only a few investigators. Weinland (95) considered that it was formed by the disintegration of protein. Bialaszewicz (12) found it to be at a maximum in the silkworm on the third day after the fourth molt. Paillet (69) thought the chitin of the silkworm cocoon was derived from glycogen. A rather complete analysis of the chitin in the various parts of *Periplaneta fuliginosa* (Serville) was made by Tauber (84).

#### REDUCING SUGARS

Glucose was not found in fly larvae, but was found constantly in adults, by Bernard (8, pp. 113-114). He concluded that glucose appeared during pupation. In a preliminary report Bataillon and Couvreur (6) stated that for a time the course of glucose in the silkworm pupae paralleled that of glycogen but the glucose continued to increase to a maximum while glycogen declined to a minimum just before emergence. Later Bataillon (5) reported that sugar did not appear in the silkworm until spinning was completed. His data are evidently those on which Bataillon and Couvreur based their conclusions. Maignon (63) agreed with Bernard in not finding glucose in the silkworm until after chrysalis formation, but stated that glucose was formed when tissues were incubated under sterile conditions under oil. His studies were extended by Vaney and Maignon (91), who found the appearance of glucose variable. They could not demonstrate glucose by the osazone test in the blood of silkworm larvae even when the gut was full of sugar. They consider that glucose was destroyed in the level of the epithelium of the intestine as soon as it reached the blood. Kawase, Suda, and Saitō (55) did not find reducing sugars in the blood of young silkworms.



Using the Hagedorn-Jensen method, Hemmingsen (46) determined the reducing substances in the blood of a number of insects, both before and after fermentation with yeast. He concluded that most of the reducing substances present were not glucose, since only a small portion was fermentable. He found that blood sugar rose after injection of insulin (47), and that insulin injected with sucrose did not hasten the disappearance of the sugar. In studies on the same insect by Wenig and Joachim (96), however, fermentable sugar disappeared within an hour after injection of insulin. The total reducing power of the blood did not change. Their normal value for the reducing power of the blood was 106 mg. per 100 cc., of which only 22.5 mg. was fermentable by yeast.

Medvedeva (65) concluded that insulin did not cause a specific reaction when injected into silkworms. Although in several experiments there was an increase in blood sugar after injection, he considered it due to a mechanical irritation set up by the process of injection rather than to the insulin. Adrenaline, on the other hand, caused a marked hyperglycemia. Blood sugar rose 71 percent above normal in the 15 minutes following injection and fell again almost as rapidly. Here, again, Medvedeva obtained a considerable hyperglycemia by the injection of water alone, and this effect also he attributed to a mechanical irritation by the syringe. He did not find a sex difference in glucose content in the hemolymph of normal imagoes.

Sturtevant (83) first found glucose in the honeybee on the third day of larval life. It increased rapidly during the feeding period but dropped off immediately after feeding ceased, and by the eighth day no reducing sugars were demonstrable. To the high concentration of sugar Sturtevant attributed the fact that *Bacillus larvae* White, the causative agent of American foulbrood, does not attack the normally feeding larvae, since the organism cannot develop in sugar solutions. Also using the honeybee, Bishop, Briggs, and Ronzoni (13) found 150 mg. of reducing substances per 100 cc. of blood. Beutler (10) found that only 2 percent of the reducing substances in adult bee blood was fermentable by yeast. After saccharose was fed the value rose. The rise was due to glucose, as in no case was fructose found. The blood sugar disappeared rapidly when it was not replenished. The queen bee had a high reducing value previous to and a low value subsequent to mating. Beutler (11) also found that the amount of sugar present was directly proportional to the energy output.

Blumenthal (17) described a micromethod for the blood analysis of insects and gave the average reducing value for the blood of *Romalea microptera* (Beauv.) (nymph) as 41.7 mg. per 100 cc., for *Melanoplus femur-rubrum* (Deg.) 35.3 mg., for *M. differentialis* (Thos.) 36.5 mg., for *Chortophaga viridijasciata* (Deg.) 34.0 mg., for *Encoptoloptus sordidus* (Burm.) 36.4 mg., and for *Popillia japonica* Newman 63.0 mg.

Frew (37) found a vigorous synthesis of glucose during the pupal period of the blowfly. Glucose was identified by its osazone. Frew suggested that the glucose originated from the histolysis of the various tissues. He was certain that glucose was not synthesized from fat either at the end of the larval period or early in the pupal period.

The blood sugar of *Deilephila euphorbiae* larvae was determined by Moklowska (68) to be 127 mg. per 100 cc. Approximately the same

value was given by Heller and Moklowska (45); they consider the amount of reducing substances present to be closely related to the stage of development.

Evans (33) found that in *Lucilia sericata* glucose fell during the first 13 days of metamorphosis and then remained constant during histogenesis. In *Tenebrio molitor* L. glucose declined from 144.2 mg. per 25 insects to 114.9 mg. during metamorphosis (34). Larvae starved for 8 days showed no carbohydrate change.

The reduction capacity of the hemolymph of several strains of silkworms was studied by Demjanowsky and Prokofjewa (26), who found that it rose sharply at each molt, fell sharply between molts, and reached a maximum at pupation. After emergence the hemolymph of the male moth showed a lower reduction capacity than that of the female. After oviposition, however, the reduction capacity of the female dropped sharply. After feeding the reduction capacity increased and reached a constant value in 5 hours. Ludwig (62) determined glucose in the Japanese beetle throughout the period of metamorphosis. In the late prepupae the glucose content was 0.50 percent, in young pupae 0.71 percent, in pupae 2 to 3 days old 0.62 percent, and at the end of the pupal period 0.84 percent.

In an attempt to find a point in the central nervous system of *Polysarcus denticauda* (Charp.) (*Orphania denticauda*) that controls glycemia, May (64) cauterized the cerebral and subesophageal ganglia and then determined the amount of reducing substances present in the blood. Although some difference between cauterized and normal insects was noted, May did not consider the difference significant. May also found that starving *Tettigonia viridissima* L. (*Locusta viridissima*) and *Carausius morosus* Brunn. (*Dixippus morosus*) for 24 hours markedly lowered the reducing value of their blood.

By isolating parts of the nervous system of fifth-instar silkworms Ermakov (32) was able to produce either hypoglycemia or hyperglycemia in the blood, depending on whether the anterior or the posterior portion of the insect was isolated. He obtained differences from normal as high as 59 percent and did not think them due to the quantity of food ingested. Crescitelli and Taylor (24) studied the changes in the reducing substances present in *Galleria mellonella* during pupation. Some glucose was present, since the osazone was isolated; but they did not consider that the entire reducing value was due to glucose. The largest amount present was 0.36 percent at pupation. No difference due to sex was observed. Yeager and Fay (98), studying the reducing value of the blood of roaches, *Periplaneta americana* (L.), that were killed under various conditions, found no significant difference between heat-killed insects, those fixed in acetic acid vapor, and those whose blood clotted under oil. There was no apparent diffusion from cell to plasma in the 15-minute clotting time, and the hemolymph from imagoes had about the same reducing power as that from large nymphs. The average value for the group of insects tested was  $62 \pm 14.4$  mg. per 100 cc., calculated as glucose, although all the reducing power was not considered to be due to glucose.

Florkin (35) determined both total and fermentable reducing substances during the metamorphosis of the silkworm. The value for fermentable reducing substances was between 10 and 49 mg. per 100 cc. of blood and followed the glycogen content as determined by

Bataillon. Florkin (36) also determined the fermentable and non-fermentable reducing substances in the blood of *Hydrous piceus* (L.) (*Hydrophilus piceus*) and found that, while total reducing substances varied between 20 and 104 mg. per 100 cc., the fermentable portion was only 5 to 31 mg.

Roy (78) could not detect glucose in the blood of larvae of *Galleria mellonella*. Babers (4) found that in the sixth-instar southern armyworm the blood had a total reducing value of 65.9 mg. per 100 cc., of which only 11.1 mg. was fermentable by yeast. Becker (7) found that the larva of *Hylotropes bajulus* (L.) did not increase in weight when glucose was added to its diet.

In a study of the reducing power of silkworm hemolymph, Kuwana (61) was not able to isolate glucosazone from the blood at any time. Using the Hagedorn-Jensen method, he found that a considerable part of the reducing power was due to a nonglucose fraction. When the sugar reagents were added to the protein-free blood filtrate and titrated immediately without the usual heating, a "cold value" was obtained. The total reducing value was obtained in the usual manner, and the total value minus the cold value gave the "hot value." Kuwana confirmed Demjanowsky and Prokofjew's finding that the total reducing value of the silkworm blood increased sharply at each molt. The increase was due to the cold value, however, and not to glucose. The portion fermentable by yeast was small, not exceeding 20 mg. per 100 cc. Upon hydrolysis with sulfuric acid, the reducing substance greatly increased, and most of the increase was fermentable by yeast. Glucose given per os caused a rise in blood sugar that reached a maximum in 3 hours and then a sharp decline to a normal value in 6 hours. In the same time the reducing power of the hydrolyzate increased. Glycolysis did not occur in the silkworm blood.

#### UTILIZATION OF CARBOHYDRATES

Kemnitz (57), from an analysis of data obtained in his study of *Gasterophilus intestinalis*, calculated that under anoxybiosis 1.39 gm. of glycogen formed 0.41 gm. of carbon dioxide, 0.2 gm. of water, and 0.1 gm. of fat as fatty acids; they combine with glycerol to form neutral fat, which is stored as reserve material. The fermentable blood sugar in the insects studied by Hemmingsen (46) did not change during starvation. Beutler (11) found that the honeybee used sugar directly without first transforming it to fat.

Known carbohydrates were fed to bees by Phillips (74), and the availability was determined from the relative length of life of the bees. Haydak (39) reported that newly emerged bees could subsist for a long time on a pure sucrose diet. Bertholf (9) also fed bees on known sugars, and found that numerous carbohydrates could be digested but that glycogen could not be utilized. Bertholf (9) has summarized the work that has been done on the carbohydrate-digesting enzymes in insects.

In the tent caterpillar Rudolfs (79) found that when the muscles were active the glycogen content was reduced, but in the silkworm Bogojavlensky (18) found little or no glycogen in the muscle. He suggested that the muscles might not use glycogen as such or that his technique might not be able to detect the quantities present. It seems probable

that the latter surmise is the correct one. The work of Acqua (2) indicates that the silkworm itself cannot digest starch; however, the mulberry leaves contained ferments that accomplished the preliminary steps so that the silkworm used the starch as soluble sugar.

The respiratory quotient obtained by Johansson (51) for yellow mealworms (*Tenebrio molitor*) that fed on flour indicates the formation of fat from carbohydrate.

Rainey (76) concluded that in *Lucilia sericata* most of the fat was derived from protein. The carbohydrate content fell from approximately 10 percent at hatching to 7 percent in the prepupae.

Timon-David (87) suggested that certain wood-eating insects might synthesize fat from pentoses, xylose in particular. Later (88) he stated that, in general, insects that consume considerable amounts of polysaccharides are very rich in fats and that these fats apparently have their origin in the sugars.

That the honeybee can convert sugar and honey into wax was first reported by Huber (49, r. 2, ch. 2) and later confirmed by Dumas and Edwards (29, 30). Abderhalden (1) found that larvae of *Anthrenus museorum* followed a seemingly normal course when fed pure silk. Carbohydrates, including chitin, were synthesized from it. Brown (21) analyzed the excreta of *Automeris io* (F.) throughout the larval period, as well as the leaves on which it fed, and decided that only hexoses and disaccharides were utilized.

In *Galleria mellonella* Taylor and Steinbach (85) found an average respiratory quotient of 0.69 for the pupal period, which might appear indicative of an exclusive oxidation of fat. These authors do not consider the explanation so simple, however, since fat oxidation is usually accompanied by oxidation of carbohydrates. They suggest the possibility of the conversion of fat to glucose and the oxidation of this carbohydrate. Although no analytical data were given, Dinulescu (27) thinks glycogen was used during nymphosis of *Gasterophilus* for the formation of fat.

Heller (40) considers that in the silkworm metamorphosis is at the expense of carbohydrates, the protein being used for the formation of the cocoon; in *Deilephila* he found the metabolism during the corresponding period to be that of fat. Heller (41) found that 50 percent of the energy content of *D. euphorbiae* larvae remained in the adult, whereas only 36 percent was present in the silkworm. In starved *Deilephila* he (42) found that the metabolism of fat required 51.7 percent of the total energy exchange, of proteins 45.5 percent, and of carbohydrates only 2.8 percent. Heller (43) found that during pupation 42.2 percent of the energy requirement was supplied by carbohydrates, 44.8 percent by fat, and 13 percent by protein. In mature butterflies during a period of inanition 964 gram-calories were used up, of which 499 were supplied by fat, 435 by protein, and only 26 by carbohydrate.

In starved larvae of *Tenebrio molitor*, Mellanby (66) found that the glycogen reserve was used up in the first 8 days; during the remaining 20 days of starvation fat was the principal reserve used. From analyses of the larval fat of *Pachymerus bacteris* (L.) (*Pachymerus dactris*), Collin (22) decided that, although some of the fat was derived from the fat ingested, a considerable portion came from the carbohydrates present in the nut on which the insect fed.

Hiratsuka (48) found that the silkworm digested 40 percent of the carbohydrate in mulberry leaves. Brown (20) fed known substances to grasshoppers and analyzed their excreta. No monosaccharides or proteins were excreted, showing complete utilization. Ten percent of the glycogen fed was excreted. In the normal diet soluble sugars and proteins were utilized while the polysaccharides were, in the main, excreted unchanged. In adult Lepidoptera Kozhantshikov (59) found that glucose and saccharose were digested. Body weight of the females increased owing to the synthesis of fat for the formation of egg yolk. Kozhantshikov considers that the carbohydrate metabolism is directly connected with fertility and that Lepidoptera adults cannot use sugar directly.

Pailot (69) suggested that glycogen was used in forming the chitin of the silkworm cocoon and that during metamorphosis the cell nuclei actively utilized glycogen. Harnisch (38) found that under anaerobiosis the larvae of *Chironomus thummi* Kieffer used mostly mesenchymal glycogen. Fat-body glycogen seems not to have been used. Increases in glycogen were noted only after feeding. According to Kato, Miwa, and Negi (54) the addition of cane sugar to the mulberry-leaf diet of the silkworm was followed by an increased digestion of protein, fat, and ash. Walter and LaHue (94) found that corn earworm adults produced only nonfertile eggs when fed a fresh-honey diet, but when freshly fermented honey was fed the eggs were 93 percent virile.

#### EXPERIMENTAL MATERIAL AND METHODS

For the purpose of the experiments larvae of the southern armyworm that were reared in greenhouse cages on turnip leaves until the end of the third instar and then placed on collard foliage are called normal larvae. The larvae pupated in moist sand, the pupae were collected, and after emergence the adults were placed in oviposition cages and allowed to feed on a dilute cane-sugar solution.

Glycogen was determined by a slight modification of Pflüger's (72) method. After digestion with 30-percent potassium hydroxide, when entire insects were used, it was necessary to clarify the solution. This was done by adding 20-percent copper sulfate solution and 10-percent sodium tungstate solution to the neutralized solution. The precipitated "brei" was removed by centrifugation and washed with water. To the combined supernatants 95-percent ethyl alcohol was added to give a solution containing 66 percent of alcohol. After the solution had stood overnight, the precipitated glycogen was centrifuged, washed with 66-percent alcohol three times, and finally taken up in 2 cc. of hot water. The insoluble material was removed by quantitative filtration through a micro asbestos filter and washed until the volume of the filtrate was 5 cc. Sufficient concentrated hydrochloric acid was added to the filtrate to make the final concentration 2.2 percent with respect to hydrochloric acid, and the solution was hydrolyzed 3 hours in the water bath, cooled, carefully brought to pH 7, and diluted to 10 cc. or larger volume depending on the amount of reducing sugar expected from preliminary runs. The reducing sugar was then determined on 5-cc. aliquots by the micro Shaffer-Hartmann method (80). The glucose value was multiplied by 0.927

to obtain the amount of glycogen. For tissue glycogen the clarification step was not needed. Blood glycogen was determined on 1-cc. samples of pooled whole blood from several insects by the method of Creveld (25), except that the reducing sugars were determined by the micro Shaffer-Hartmann method. Blood sugars were run by the micro Shaffer-Hartmann-Somogyi technique as described by Peters and Van Slyke (71).

For the feeding experiments a paste was made by heating 2 gm. of glucose, 1 gm. of cornstarch, and 15 cc. of water just to the thickening point. Sandwiches were then made from disks of turnip leaves, cut out with a No. 10 cork borer, and portions of this paste containing approximately 12 mg. of glucose. One sandwich was offered to a single larva, and only those larvae that consumed an entire sandwich in the course of 30 minutes were used. After ingestion of a sandwich, the larva was placed on turnip foliage until used.

The term "tissue," unless otherwise designated, refers to the material remaining after removal of the blood and alimentary tract. Where blood analyses were made, the larvae were killed and their blood was partially fixed by immersing them for 1 minute in water at 60° C. The blood was collected after the snipping of a leg. After removal of the blood, the alimentary tract was dissected out and the remaining tissue dried in a weighed container at 80°. Control experiments showed no apparent loss of glycogen during the drying period. The dried tissues were weighed and analyzed individually for glycogen by the method mentioned above.

#### NORMAL VARIATION IN GLYCOGEN CONTENT

Beginning with eggs and ending with long-lived adults, analyses were made each day to determine the normal variation and the period of greatest glycogen accumulation. For the egg analyses several egg masses that were deposited within 2 hours of each other were used. The time of hatching was considered the beginning of larval life. The entire egg mass usually hatched within a short time after the first larvae hatched, and those larvae that hatched within 1 hour were grouped and reared together. Once a day the required number were removed, dried, and weighed, and the entire insects were analyzed for glycogen. The results of these analyses are shown in figure 1. Each point on the curve represents the average of the analyses of several groups of insects of the age indicated. That there is considerable difference in the glycogen content of male and female moths is clearly shown in figure 2.

The fat body has long been considered the storehouse of glycogen for the insect. This conclusion has been based almost entirely on histological evidence. Of the amount actually present, however, no record has been found. The fat body was therefore removed from mature sixth instars as completely as was conveniently possible with small forceps. The fat body from 10 larvae was combined, and when it was dry glycogen was determined in the usual manner. The weight of dry fat body obtained from 10 insects varied between 26.390 and 49.780 mg. and the percentage of glycogen between 17 and 27, with an average of 23.32 percent dry weight. The fat body is truly rich in glycogen.

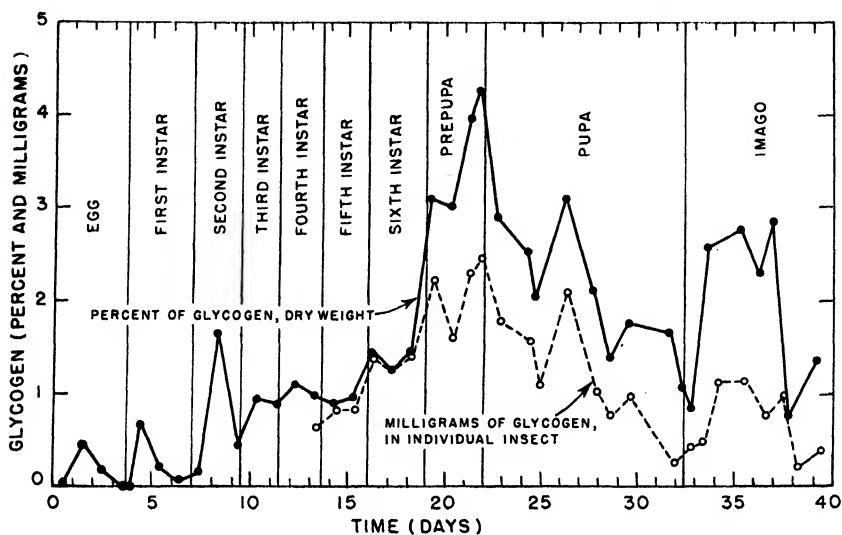


FIGURE 1.—Glycogen content of *Prodenia eridania* as indicated by daily analyses of entire insects throughout the life cycle.

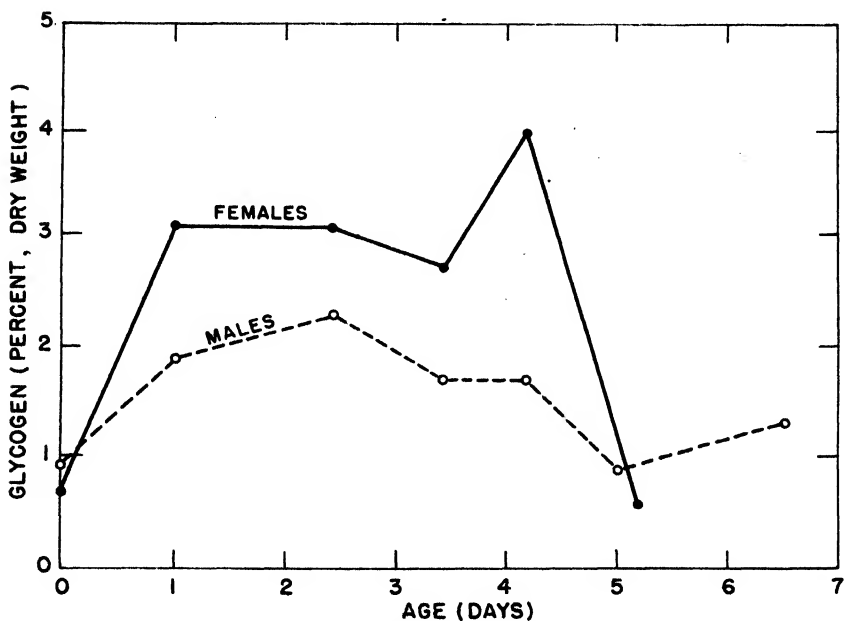


FIGURE 2.—Glycogen (dry weight basis) contents of male and female adults of *Prodenia eridania*.

## EFFECTS OF GLUCOSE INGESTION

After the larvae had ingested glucose sandwiches, the hemolymph was analyzed for reducing substances at varying intervals beginning 15 minutes after normal feeding commenced. Analyses were made on the blood of individual insects, and each point on the curve in figure 3 represents the average reducing value, calculated as glucose, of approximately 25 insects.

When the blood from insects that had ingested a single sandwich was analyzed for glycogen, no definite variation from the normal glycogen content was found. In two instances a tremendous rise was obtained, the blood glycogen jumping to 69 and 178 mg. per 100 ml. of blood. Since in about 25 other tests no rise in glycogen content was found, it is believed that the high values obtained were due to

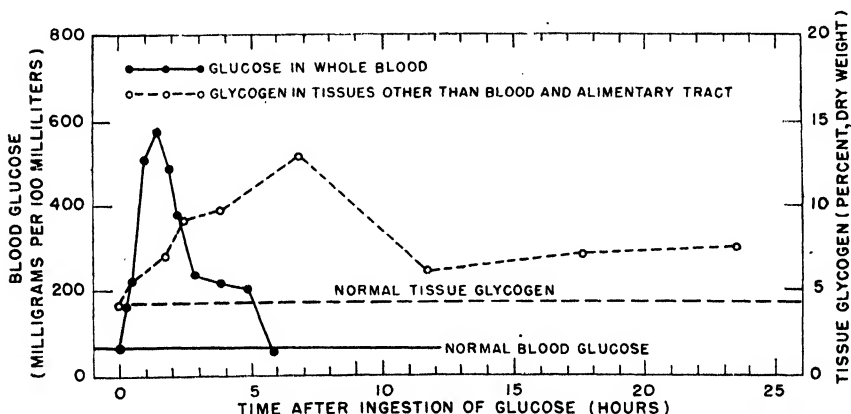


FIGURE 3. Glucose and glycogen content of glucose-fed instars of *Prodenia eridania*.

some other cause, possibly incomplete digestion with potassium hydroxide. However, when larvae were allowed to feed overnight on turnip leaves smeared with glucose-starch paste, there was a definite increase in blood glycogen. The normal glycogen content of the blood of sixth-instar southern armyworms is between 1.3 and 4.5 mg. per 100 ml. The average of 10 analyses of 1-ml. samples was 3.29 mg. per 100 ml. Four samples of pooled blood from larvae that had fed on glucose overnight contained, respectively, 33.4, 17.42, 13.46, and 19.9 mg. of glycogen per 100 ml. The average reducing value of these samples was 133 mg., as glucose, per 100 ml. of blood, of which 63 mg. was fermentable by yeast.

In one experiment the cells and plasma were analyzed separately. The heat-fixed blood was centrifuged for 10 minutes at approximately 2,000 revolutions per minute, and the plasma was drawn off with a fine pipette. The cells were then washed twice with saline and the wash solutions combined with the plasma. In the cells 16.5 mg. of glycogen per 100 ml. was found and in the plasma 0.92 mg. It is probable that the small quantity found in the plasma was originally in the cells and was removed by the drastic treatment to which they



were subjected. After removal of the blood the alimentary tracts of these insects were dissected out, and glycogen was determined in the remaining tissue. The tissue from each insect was analyzed separately, and the results were averaged to obtain the point shown on the tissue-glycogen curve in figure 3. For the tissue of normal larvae with an average glycogen content of 4.26 percent, the standard deviation was 1.81. Four hours after the larvae had consumed a glucose sandwich the average tissue glycogen was 9.92 percent, with a standard deviation of 2.29. The standard deviation was not calculated for other points on the curve. The tissues from the larvae that had fed overnight on glucose contained 14.75 percent of glycogen, dry weight, and the tissue from individual larvae contained on an average 6.28 mg. of glycogen.

Hemolymph from normal sixth instars contained on an average 64.9 mg. of reducing substances, calculated as glucose, per 100 ml. of blood. Of this quantity only 11.1 mg. was fermentable by yeast, and was presumed to be glucose. Many investigators have reported similar low fermentable values in the insects tested, but many have also reported that in larval blood the glucosazone test was always negative. Upon examination under the microscope, however, glucosazone crystals were found in the blood of sixth-instar southern armyworms after treatment in the following manner. Three ml. of pooled blood from insects killed by immersion in water at 60° C. for 1 minute was diluted to 5 ml. with distilled water. Proteins were precipitated by the addition of 0.8 ml. of an acid cadmium sulfate solution (13 gm. of cadmium sulfate and 63.5 ml. of normal sulfuric acid per 100 ml.) and 1.1 ml. of normal sodium hydroxide (67). After dilution to 10 ml. and centrifugation, the supernatant was poured onto a mixture of 0.4 gm. of phenylhydrazine hydrochloride and 0.6 gm. of sodium acetate that had been finely ground together in a mortar. The test tube containing the mixture was protected from evaporation and placed in a boiling-water bath for 2 hours. The source of heat was then removed and the tube left in the bath until the latter had cooled to room temperature. Microscopic examination then showed the presence of widely scattered but definite clusters of glucosazone crystals.

Blood from insects that had ingested a glucose sandwich an hour before the blood was drawn also gave a positive glucosazone reaction. In this case, however, the glucosazone crystals were present in large numbers soon after the heating with phenylhydrazine was begun.

#### DISCUSSION

In general the curve showing the daily variation in the glycogen content of the southern armyworm is similar to the curves obtained by most other investigators for other insects. With the exception of Külz (60), who failed to find glycogen in the eggs of the fly *Calliphora vomitoria*, they seem generally agreed that the eggs of the various insects studied contain appreciable quantities of glycogen soon after deposition and that the glycogen then decreases steadily until minute quantities, if any, are found just before hatching. In the southern armyworm larvae glycogen appears soon after feeding commences, and increases to a peak at pupation. This also is in general agreement with other insects studied.

The time of greatest accumulation has been given as at pupation or just before or after. It is not surprising that a slight disagreement exists, if for no other reason than the extreme difficulty in defining the instant of pupation exactly. It is evident, however, that glycogen continues to be formed after the larva has ceased feeding. That this is an actual increase in the quantity of glycogen, and not a percentage increase due to loss of weight, is shown by the analyses of individual insects. Whether this glycogen is formed from soluble foodstuffs remaining in the alimentary tract before its complete evacuation or from autolyzing tissues is not clear. During the pupal period, as one would expect, there is a steady decline in the quantity of glycogen present. The chemical changes involved are not yet understood. The glycogen content rises again in the adult, and there is a distinct sex difference, the females having the highest glycogen content. Although little experimental evidence is presented to support the theory, it seems probable that the additional glycogen is the source of the energy required for egg production and of the glycogen found in the egg. It is not known whether the glycogen is deposited in the egg as such or whether the various cells synthesize it from other material. However, the evidence is overwhelming that glycogen plays a very important part in the general metabolism of insects.

At the time these experiments were started, very little quantitative experimental evidence was available that showed the type of foodstuff from which insects derive their glycogen supply. Most conclusions were based on the disappearance of one substance and a corresponding increase in another. After the completion of these experiments, however, the work of Kuwana (61) came to the writer's attention. This investigator, using the silkworm, has performed several experiments similar to those made by the present writer. The two curves for blood sugar after the feeding of glucose are very similar in shape. Kuwana obtained a peak value of 594 mg. of reducing substances as glucose in 3 hours, whereas the peak of 581 mg. obtained by the writer came in 1½ hours. The rapid rise and decline, however, were similar. In addition, Kuwana found a tremendous increase in reducing substances after acid hydrolysis of the blood of glucose-fed larvae, but he did not consider this to be due entirely to glycogen. The writer, of course, did not get an increase in blood glycogen after feeding only one sandwich. However, Kuwana apparently hydrolyzed whole blood with acid without the preliminary alkali treatment and alcohol precipitation used by the writer. It therefore seems probable that only a small part, if any, of the increase Kuwana found was due to glycogen. He did not follow the course of tissue glycogen after glucose ingestion. In the writer's experiments tissue glycogen increased definitely within a short time after glucose was fed, and the peak of glycogen formation came approximately 4½ hours after the blood-reducing value had reached its maximum. It is interesting to note that the rise in blood glycogen came only after long feeding on glucose-smeared leaves, as if it were an overflow into the blood after the tissues had reached a saturation point. The pH value of most insect blood is on the acid side. That of the southern armyworm is 6.53, and this acid condition may well retard glycogen formation and hasten its utilization in the blood.

The southern armyworm, therefore, possesses an efficient glycogen-synthesizing mechanism by which glucose can be readily transformed into glycogen. The site of this transformation is not known, but if one reasons by analogy with vertebrates, which of course is a risky procedure, the site of formation is also the site of the greatest glycogen concentration. If this is correct, then at least one function of the fat body is the same as that of the vertebrate liver.

The failure of other workers to identify glucose in larval blood by the osazone test has probably been due to their failure to use a large enough sample. Although the glucosazone test is very sensitive, in small samples the presence of glucosazone crystals may be easily overlooked, since at best there can be only a few crystals present when the sample contains only 0.05–0.2 mg. of glucose per milliliter.

### SUMMARY

The glycogen content of the southern armyworm (*Prodenia eridania* (Cram.)) has been studied as part of an investigation of the carbohydrate metabolism of insects. A comprehensive summary of the literature on the carbohydrate metabolism of insects is included as a background for this study. This review shows that glycogen is widely distributed among insect species. Fats and proteins have both been suggested as the source of glycogen, but there is greater evidence that most of the glycogen comes from other carbohydrates. Glycogen and protein have been suggested as the source of chitin. Glucose has been found free in the hemolymph in only small quantities. The quantity of blood glucose seems to be related to the energy output. It is shown that insects are able to digest and utilize many carbohydrates.

In the experimental work with the southern armyworm the normal variation in glycogen content was first studied by analyzing entire insects at daily intervals throughout their life cycle. The effect of glucose ingestion was then studied by feeding sixth instars turnip-leaf sandwiches containing glucose and determining the blood glucose, blood glycogen, and tissue glycogen after varying intervals.

The results on normal variation were in general agreement with the findings of previous investigators on other insects. Glycogen was present in the egg, but disappeared at hatching, reappeared in the larvae after feeding had begun, and rose to a maximum at pupation; during pupation glycogen fell steadily and then rose again sharply in the adult. The female moths consistently contained more glycogen than the males.

Analyses of the fat body of mature sixth instars showed it to contain on an average 23.32 percent (dry weight) of glycogen.

In the feeding experiments the blood glucose rose sharply within 15 minutes, reached a maximum in  $1\frac{1}{2}$  hours, and returned to normal in 6 hours. Blood glycogen did not rise after one sandwich was fed. Tissue glycogen rose to a maximum in 7 hours after ingestion of a glucose sandwich.

Glucose was demonstrated in normal larval blood by the osazone test.

It is concluded that the southern armyworm possesses an efficient glycogen-forming mechanism by which glucose is transformed into glycogen. The chief site of glycogen formation appears to be the fat body.

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# CALCIUM REQUIREMENTS OF GROWING PIGS<sup>1</sup>

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## INTRODUCTION

The role of calcium and phosphorus in animal nutrition has received much attention in recent years, and many studies have been made to determine the need of these minerals in the nutrition of the pig. Much of the work has dealt with deficient rations and means of correcting the deficiencies. Recently the individual minimum requirements of these minerals have received the attention of investigators. Vitamin D in connection with these requirements has also been investigated (3, 6, 9).<sup>3</sup>

## REVIEW OF LITERATURE

The requirements of phosphorus in the presence of vitamin D for the growing pig have been worked out by Aubel, Hughes, and Lienhardt (1), who place the minimum value between 0.27 and 0.30 percent when the calcium level is 0.8 percent. Spildo (11) and Axelsson (2) place the minimum value at 0.38 and 0.20 percent, respectively, and Mitchell and McClure (10) estimate it at 0.37 percent for a 30-pound pig and at 0.18 percent for a 200-pound pig. The lowest level fed by Dunlop (6) was 0.53 percent, at which level, with adequate calcium and vitamin D, he obtained satisfactory development. He does not report feeding a ration containing less than 0.53 percent phosphorus, and this, therefore, cannot be said to be a minimum level. Thus the minimum percentages of phosphorus required in the ration as so far determined are generally in fairly close agreement.

The requirement of calcium when vitamin D is supplied is reported by Dunlop (6) to be 0.34 percent, and he found a normal development of blood, bones, and body growth at this level whether or not vitamin D was supplied. Dunlop places considerable stress on the calcium-phosphorus ratio. He says, "A calcium level adequate at one ratio of calcium-phosphorus may be inadequate at another." In ascertaining his minimum calcium level Dunlop fed varying amounts of calcium with phosphorus levels at 0.53 percent or higher.

Other investigators have also reported the calcium requirements of pigs in the presence of vitamin D. Spildo (11) obtained satisfactory results with pigs which received 0.34 percent of calcium and 0.41 percent of phosphorus. Theiler and his associates (12) conducted experiments primarily on the effect of rations deficient in phosphorus or calcium or both. Their rations were deficient in calcium at a level of 0.10 percent and were adequate at a level of 2.0 percent. They did not feed any pigs a ration at a 0.30-percent level of phosphorus. Axelsson (2) estimates the percentage requirement of calcium at 0.37. His phosphorus level for this level of calcium was

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<sup>3</sup> Italic numbers in parentheses refer to Literature Cited, p. 542.

0.20 percent. Bohstedt (5) reports the most satisfactory results with levels of calcium at 0.32 and 0.41 percent in the presence of 0.28 and 0.29 percent of phosphorus. Aubel and his associates (1) produced satisfactory pigs with 0.8 percent of calcium and 0.3 percent of phosphorus. The calcium requirements in their study, however, were not directly under scrutiny. A level of 0.8 percent was fed so as to be sure that the pigs received ample calcium. Mitchell and McClure (10), on the basis of the composition of the body, estimate the requirements of calcium for a 30-pound pig to be 0.53 percent and state that it decreases gradually as the pig increases in weight, so that a 200-pound pig requires only 0.20 percent of calcium.

From these observations it would appear that with the exception of the work of Bohstedt (5), for which detailed data on blood and bone composition are not available, the minimum calcium requirements for growing pigs fed 0.3 percent of phosphorus, has not been determined. Aubel and his associates (1) found 0.3 percent of phosphorus to be the minimum level, a value which generally is approximated by the findings of other workers.

In view of the variations in the calcium requirements of pigs reported in the literature it seemed desirable to examine this question further. It is, therefore, the purpose of this paper to give information not only on the effects of a deficiency of calcium in the ration on the development of young pigs, but also to show the minimum requirements of calcium necessary to insure normal growth and bone and blood formation, when the phosphorus level is maintained at 0.3 percent and ample vitamin D is supplied.

#### EXPERIMENTAL PROCEDURE

Similar groups of pigs were fed rations which contained the same amounts of digestible nutrients, minerals, and vitamins, but different amounts of calcium. The response of the pigs, as reflected in body and bone growth, utilization of feed, and blood composition, was taken as the criterion for determining the amount of calcium necessary to meet the requirements for normal development.

The animals in each experiment were a uniform-appearing lot of purebred Hampshire pigs about 9 weeks of age and weighing about 40 pounds when the experiments were started shortly after weaning. Before being put on test the pigs were immunized against hog cholera and treated for roundworm. All pigs were housed in a well-lighted and well-ventilated building provided with individual concrete paved pens 6 by 8 feet in size. Each pen was provided with an outside concrete paved exercise area fenced with wire. The outside area allowed the pigs access to the direct rays of the sun and thus they were exposed to ultraviolet rays in considerable amount.

The animals were fed individually twice a day, the amount being determined by the normal-appearing pig with the smallest appetite. This arrangement insured all the pigs ingesting the same amount of food, so that differences in gains in weight and growth could not be attributed to variations in intake of food.

The basal ration consisted of 74 percent of pearl hominy, 10 percent of tapioca roots, 10 percent of blood meal, 4 percent of dehydrated alfalfa-leaf meal, 1.5 percent of dried brewer's yeast,<sup>4</sup> and 0.5 percent of iodized salt. Each pig received 5 cc. of cod-liver oil per

<sup>4</sup> This yeast was the Red Label brand of the Vitamin Food Company, Inc., New York City.

day. Enough monocalcium phosphate was used to raise the phosphorus content to 0.30 percent in all lots except lot 4, in experiment 2. In this lot, monosodium phosphate was used so that the calcium could be kept at 0.10 percent. Supplemental additions of calcium to vary the calcium level were made in the form of calcium carbonate. This ration was thought to contain, with the exception of the calcium, an adequate amount of all the elements known to be necessary for the normal growth of a pig.

The pigs were weighed every 28 days throughout the experiments. A determination of the calcium, inorganic phosphorus, and phosphatase in the blood was made at the beginning of each experiment and at the end of each 28-day period. A single sample of blood was drawn from the tail for these determinations. The Wang (13) method was used for the determination of calcium; phosphorus was determined by the Fiske and Subbarow (7) method, and phosphatase by the Bodansky (4) method.

The feeding period for each experiment was 24 weeks, at the end of which time the animals were killed and a pathological inspection of the carcasses was made. The femur and humerus bones of the right side of each pig were saved for chemical and physical analyses. A 75,000-pound Southwark-Emery hydraulic compression testing machine was used to determine the breaking strength of the bones.

#### EXPERIMENT 1

In experiment 1, conducted from December 2, 1938, to May 19, 1939, three lots of pigs were used. Lot 1 contained five pigs, and lots 2 and 3 four pigs each. The levels of calcium fed were 0.6 percent for lot 1; 0.8 percent for lot 2; and 1.0 percent for lot 3. The 1.0-percent level was included in order to observe the effect of an excess over the previously observed satisfactory level of 0.8 percent (1), and the 0.6-percent level was fed in an effort to locate a minimum level.

The growth, feed records, and composition of the blood and bone are shown in table 1, and the growth curves in figure 1.

TABLE 1.—Effect of different calcium intakes, at higher levels, on the growth, the feed utilization, the blood composition, and bone formation in the pigs in experiment 1

Lot No.	Pigs in lot	Calcium in ration	Phosphorus in ration	Average initial weight	Average final weight after 24 weeks' feeding	Average daily gain	Average daily feed	Feed required per 100 pounds of gain in weight
	Number	Percent	Percent	Pounds	Pounds	Pounds	Pounds	Pounds
1	5	0.60	0.30	39.6	237.0	1.17	4.28	365.8
2	4	.80	.30	40.4	237.5	1.17	4.28	365.8
3	4	1.00	.30	41.6	236.7	1.16	4.28	368.9

Lot No.	Blood analysis at end of experiment			Average specific gravity of humeri	Average breaking strength of humeri	Bone analysis (dry fat-free basis)		
	Average calcium in 100 cc. of serum	Average inorganic phosphorus in 100 cc. of serum	Average phosphatase in serum			Average ash in femurs and humeri	Average calcium in femurs and humeri	Average inorganic phosphorus in femurs and humeri
	Milli-grams	Milli-grams	Units		Pounds	Percent	Percent	Percent
1	13.4	7.6	7.88	1.311	1.230	54.33	21.19	9.98
2	12.2	8.4	9.15	1.314	1.393	52.03	20.85	10.07
3	13.6	8.0	8.10	1.260	1.345	52.40	21.05	9.87

## RESULTS

All the pigs in experiment 1 developed normally. It can be seen from the data in table 1, and from the curves in figure 1, that they were similar in all respects. They made almost the same daily gains, consumed the same amount of feed, had practically identical efficiency in utilizing their feed, had normal blood-serum calcium, phosphorus, and phosphatase content, and the bone formation was

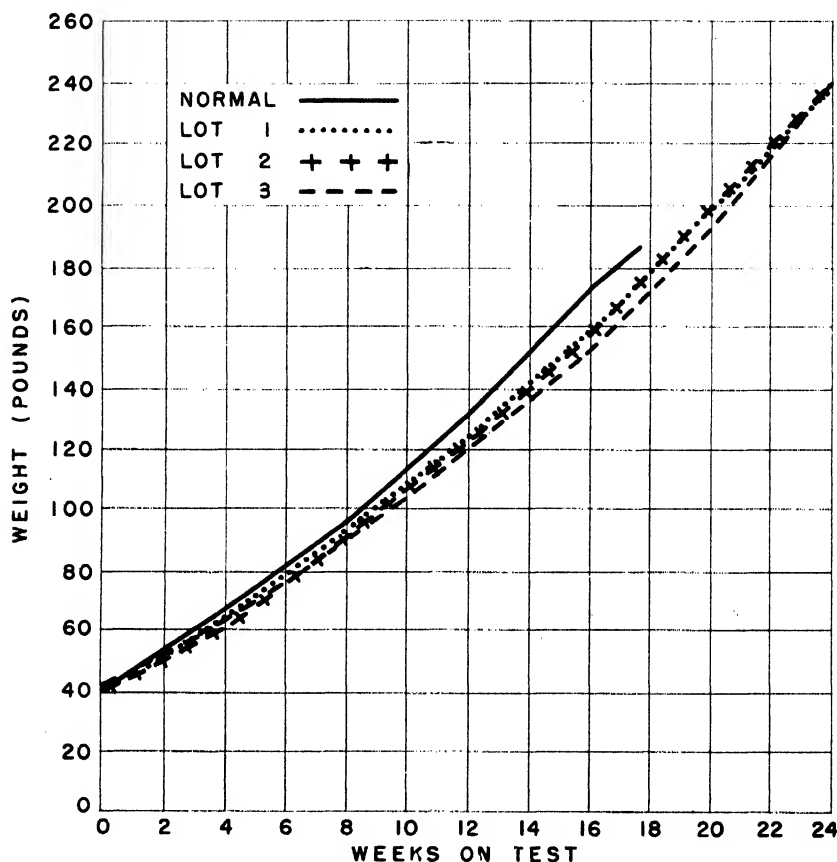


FIGURE 1.—Weights of pigs of lots 1, 2, and 3 with calcium intakes of 0.60, 0.80, and 1 percent, respectively, as compared with the weights of normal pigs. The curve for normal animals was drawn from data supplied by the Division of Animal Husbandry of the University of California and represents the average of the records of 457 pigs that had been on excellent rations (8).

normal as evidenced by the ash content and breaking strength of the bones. It is apparent from these data that a calcium level as high as 1.0 percent, and a level as low as 0.6 percent can be fed without abnormal results when ample vitamin D is supplied, and the phosphorus level is 0.3 percent of the ration. Under the conditions of this experiment, the requirements of calcium for the growing pig do not exceed 0.6 percent, the lowest level fed.

## EXPERIMENT 2

The second experiment, conducted from December 6, 1939, to May 22, 1940, included four lots of pigs. Lot 4 contained three pigs, lot 5 five pigs, and lots 6 and 7 four pigs each.

In order that satisfactory comparisons might be made, experiment 2 was conducted with pigs of the same size and origin and in precisely the same manner as the first experiment. Lot 4 of experiment 2 received 0.10 percent of calcium; lot 5, 0.25 percent; lot 6, 0.41 percent; and lot 7, 0.55 percent. The high calcium level of 0.55 percent in this experiment was fixed just under the low level of lot 1 in the first experiment.

It was thought that the level of 0.55 percent in lot 7 would be near enough to the 0.6 percent of lot 1, experiment 1, to serve as a check on that level. In order to facilitate comparison, it was also planned in this experiment to control the intake of feed so that it would be similar to the amount ingested daily by the pigs in the first experiment.

The growth, feed records, and composition of the blood and bone of the second experiment are given in table 2 and the growth curves in figure 2.

TABLE 2.—Effect of different calcium intakes, at lower levels, on the growth, the feed utilization, the blood composition, and bone formation in the pigs in experiment 2<sup>1</sup>

Lot No.	Pigs in lot	Calcium in ration	Phosphorus in ration	Average initial weight	Average final weight after 24 weeks of feeding	Average daily gain	Average daily feed	Feed required per 100 pounds of gain in weight
	<i>Number</i>	<i>Percent</i>	<i>Percent</i>	<i>Pounds</i>	<i>Pounds</i>	<i>Pounds</i>	<i>Pounds</i>	<i>Pounds</i>
4	3	0.10	0.31	41.5	182.3	0.83	3.79	456.6
5	5	.25	.30	40.8	219.0	1.06	4.23	399.0
6	4	.41	.30	41.4	232.5	1.13	4.30	380.5
7	4	.55	.30	41.9	232.7	1.13	4.27	377.8

Lot No.	Blood analysis at end of experiment			Average specific gravity of humeri	Average breaking strength of humeri	Bone analysis (dry fat-free basis)		
	Average calcium in 100 cc. of serum	Average inorganic phosphorus in 100 cc. of serum	Average phosphatase in serum			Average ash in femurs and humeri	Average calcium in femurs and humeri	Average inorganic phosphorus in femurs and humeri
	<i>Milli-grams</i>	<i>Milli-grams</i>	<i>Units</i>		<i>Pounds</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>
4	12.11	8.6	7.0	1.147	.733	51.63	19.30	10.03
5	12.25	8.6	6.5	1.252	1.085	57.36	23.40	10.59
6	12.35	8.9	6.9	1.293	1.132	60.80	23.15	11.07
7	12.72	9.0	5.8	1.323	1.230	61.76	23.10	11.41

<sup>1</sup> Data for 1 pig in lot 5 not included.

## RESULTS

All pigs in experiment 2 had smooth coats, good appetites, were thrifty, and made good gains throughout the experiment with the following exceptions:

In lot 4 (0.10 percent of calcium) all the pigs showed poor appetite. They developed severe diarrhea almost at the beginning of the ex-

periment and it lasted until the ninth week. During this time the pigs had poor appetites except on two or three occasions when their appetites returned and diarrhea ceased for short periods. They became coarse-haired and were nervous. After the tenth week, two of the pigs recovered to some extent and ate as much as the pigs in the other lots, so that by the end of the experiment they were fairly

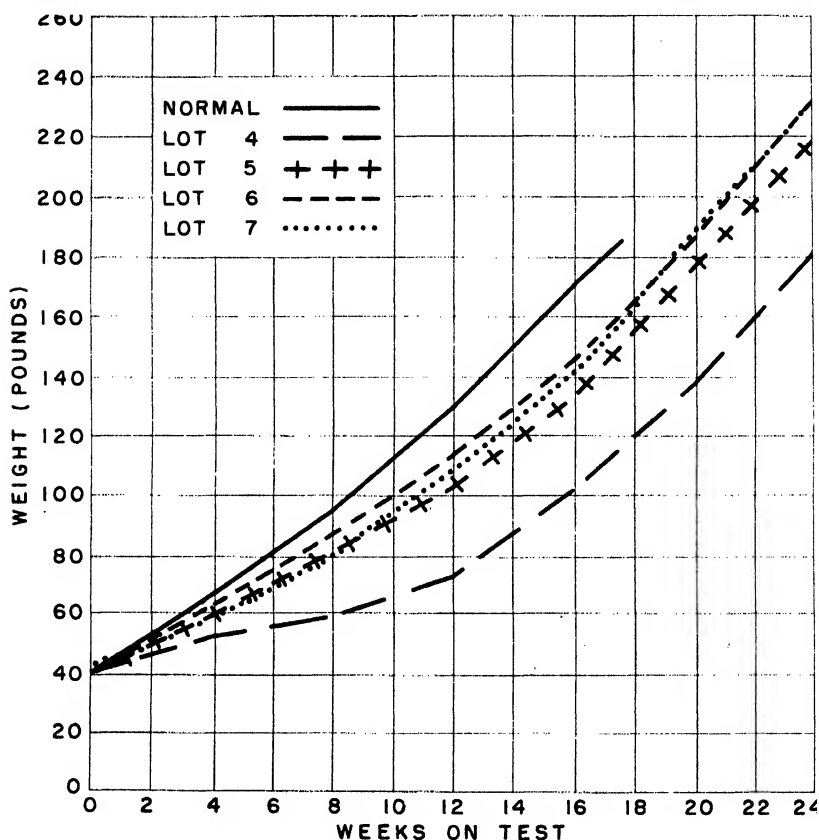


FIGURE 2.—Weights of pigs of lots 4, 5, 6, and 7 with calcium intakes of 0.10, 0.25, 0.41, and 0.55 percent, respectively, as compared with the weights of normal pigs. The curve for normal animals was drawn from data supplied by the Division of Animal Husbandry of the University of California and represents the average of the records of 457 pigs that had been on excellent rations (8).

smooth in coat, quite thrifty in appearance, and appeared normal except in weight. The third pig in this lot lost appetite the fifth week, developed severe diarrhea, and showed symptoms of weakness in the hindquarters. This pig did not recover its appetite until the twenty-first week. At slaughtering, the pigs in this lot had soft bones. It is significant that all the pigs on this low-level calcium ration exhibited the same calcium-deficiency symptoms described by Theiler and his associates (12). It is also significant that these pigs showed calcium-deficiency symptoms early and that after about 3

months of feeding they improved, especially in food consumption and thriftiness.

Two of the five pigs in lot 5, receiving 0.25 percent of calcium, gave indications of impaired appetite, unthriftiness, and diarrhea early in the experiment; pig No. 4 by the end of the third week, and pig No. 5, two weeks later. By the seventh week these pigs were obviously suffering from some deficiency. It was thought that possibly there might be a deficiency of water-soluble vitamin B in the ration. The yeast was accordingly increased to 5 percent in the ration of pig No. 4, and 5 mg. of nicotine acid was given orally each day to pig No. 5. These alterations in the feed were continued for 4 weeks, but there was no improvement in the condition of the pigs. By this time their appetites were very poor, they had developed severe diarrhea, and No. 4 was unsteady on his legs and slightly nervous (fig. 3, *A* and *B*).

By the end of 3 months pig No. 4 was paralyzed in the hindquarters and ate his food in a lying position. When it was apparent that he would not recover he was killed. An autopsy did not show any



FIGURE 3.—Two views of pig No. 4, lot 5, experiment 2: *A*, After 8 weeks on ration containing 0.25 percent of calcium. Note the braced hind legs of this pig by means of which he keeps on his feet. *B*, After 12 weeks feeding, showing lying position and paralyzed hindquarters.

lesions that could account for the paralysis or the emaciated condition. The data in table 2 do not include those for pig No. 4.

Pig No. 5 showed much the same symptoms as pig No. 4, but never refused feed for more than 2 or 3 days at a time. From the tenth week until the end of the experiment this pig ate as much feed, most of the time, as the other pigs in the experiment. Although his appetite and appearance improved he never was as sleek-haired and thrifty as the other three pigs in this lot. Pigs Nos. 4 and 5 had soft bones when slaughtered.

Two of the five pigs in lot 5, which received 0.25 percent of calcium, showed calcium-deficiency symptoms early in the experiment, but one had recovered to a considerable extent by the end of the experiment. The other three seemed normal in appetite and growth, but at slaughter their bones were found to contain less ash and to break more easily than the bones of pigs receiving a higher level of calcium. It would seem, therefore, that the 0.25-percent level of calcium as fed in this lot might be close to the border line of calcium requirements for growing pigs.



Symptoms of calcium deficiency were not observed in lot 6 (0.41 percent of calcium) and in lot 7 (0.55 percent of calcium). The pigs in both lots developed and grew normally and had normal bones and blood as compared with lots 1, 2, and 3 of the first experiment.

#### DISCUSSION OF RESULTS

From the results obtained by feeding different levels of calcium it is evident that under the conditions prevailing in these experiments a level of 0.10 percent of calcium (lot 4, experiment 2) was inadequate for the normal development of pigs; they failed to gain normally, to utilize their feed efficiently, and to develop strong bones with normal ash content.

A 0.25-percent calcium level (lot 5, experiment 2) appeared to be near the border line or slightly below the calcium requirement. Two of the five pigs developed abnormally; the remaining three made normal gains in weight, but their bones contained a subnormal percentage of ash associated with a lowered breaking strength.

All pigs fed a ration containing 0.41 percent or more of calcium appeared to develop normally in every respect. Furthermore the feed consumption, utilization of feed, the composition of the blood, and the formation of the bone were similar with calcium levels ranging from 0.41 to 1.0 percent. These results indicate that raising the calcium level above 0.41 percent does not improve a feed for growing pigs.

Therefore, under the conditions of this experiment, when the level of phosphorus is 0.3 percent and adequate vitamin D is supplied, it would seem that a level of 0.25 percent of calcium is slightly below the minimum requirements while 0.41 percent is definitely adequate for the normal development of pigs.

#### EFFECT OF LOW-CALCIUM RATIONS ON THE BLOOD COMPOSITION

The effect of low-calcium rations on the composition of the blood has been reported by other investigators. Theiler and his associates (12) stated that the serum calcium was not significantly affected by the calcium of the ration. The findings herein reported are not entirely in agreement with this statement.

The data for the composition of the blood of the pigs in experiment 2 are shown in table 3. An inspection of these data shows that the serum calcium values of the lot 4 pigs and pigs Nos. 4 and 5 of lot 5, which showed calcium-deficiency symptoms, began to show a downward trend after 28 days on the low-calcium ration. This decrease in serum calcium continued for about 56 days, after which it increased, until at the end of the experiment the values were normal.

In the pigs that received an amount of calcium as low as 0.10 percent the blood calcium was not depressed below 8.92 mg. per 100 cc. of serum. It is not surprising, therefore, that low-calcium tetany was not observed in the pigs on a low-calcium diet in this experiment since such symptoms do not appear until blood calcium values fall below this value. It is worthy of note also that only the pigs showing slightly depressed serum calcium manifested any clinical evidence of calcium deficiency, such as loss of appetite, unthrifty appearance, and stiffness. These symptoms disappeared as the serum calcium returned to normal.

The reestablishment of serum calcium to normal after it had been slightly depressed would indicate that although a pig is receiving too little calcium in its diet, the system is able to maintain, apparently at the expense of bone calcium, almost a normal content of calcium in the blood. It should be emphasized, however, that ample vitamin D was provided. It seems surprising that pigs receiving a calcium-deficient diet sufficient to affect the appetite and produce the other clinical symptoms of calcium deficiency, are able to adjust themselves and subsequently recover their appetite and consume enough feed with its attendant amount of calcium to bring about satisfactory gains in body weight and a normal blood picture.

The data in table 3 also show that the inorganic phosphorus of the serum was little affected by the low-calcium rations.

TABLE 3.—Calcium, inorganic phosphorus, and phosphatase content of 100 cc. of the blood serum from 5 pigs of lots 4 and 5, which showed calcium-deficiency symptoms, as compared with the means of these values for the pigs in lots 6 and 7, which received an adequate supply of calcium

[Determinations made at 28-day intervals]

Lot No.	Pig No.	Initial			28 days			56 days			84 days			
		Cal-cium	Inor-ganic phos-phorus	Phos-phatase	Cal-cium	Inor-ganic phos-phorus	Phos-phatase	Cal-cium	Inor-ganic phos-phorus	Phos-phatase	Cal-cium	Inor-ganic phos-phorus	Phos-phatase	
Calcium deficient:		Milli-grams	Milli-grams	Units	Milli-grams	Milli-grams	Units	Milli-grams	Milli-grams		Milli-grams	Milli-grams	Units	
4	{	1	12.40	8.0	3.6	13.36	9.0	3.4	10.52	8.1	6.1	8.92	8.0	5.4
		2	11.20	8.3	2.2	12.72	9.0	2.9	10.92	7.1	6.2	8.92	6.8	5.6
		3	11.66	8.0	2.8	13.14	8.9	2.5	11.24	7.0	5.0	9.63	6.2	2.1
		4	12.02	7.0	3.3	12.92	8.8	2.8	10.86	7.7	7.3	11.56	9.5	2.3
5	{	5	12.29	8.2	4.5	13.36	9.3	2.5	10.82	8.8	7.2	9.63	6.9	4.3
		6	12.40	8.2	1.4	13.56	9.8	3.8	12.24	10.2	9.8	11.31	8.8	5.8
		7	12.31	8.0	.9	13.56	9.5	3.6	14.82	10.0	11.3	11.55	7.9	6.7
		8	11.65	9.0	2.1	12.72	9.3	3.9	11.10	9.4	7.7	11.31	8.7	6.0
Calcium adequate:														
6	(2)	11.70	8.0	2.0	12.78	9.2	4.6	12.49	9.4	9.8	10.77	7.9	8.1	
7	(2)	11.62	7.5	2.1	12.92	7.8	3.9	12.85	7.8	9.2	11.37	7.6	6.9	

Lot No.	Pig No.	112 days			140 days			168 day			
		Cal-cium	Inor-ganic phos-phorus	Phos-phatase	Cal-cium	Inor-ganic phos-phorus	Phos-phatase	Cal-cium	Inor-ganic phos-phorus	Phos-phatase	
Calcium deficient:		Milli-grams	Milli-grams	Units	Milli-grams	Milli-grams	Units	Milli-grams	Milli-grams	Units	
4	{	1	10.27	9.6	10.1	10.47	8.1	7.9	11.90	8.9	6.1
		2	11.13	8.8	9.6	10.41	8.0	11.7	12.26	9.0	6.1
		3	10.70	7.0	6.7	9.86	7.1	11.2	12.17	7.9	8.9
		4	11.23	8.5	11.2	11.40	7.9	7.8	(1)		
5	{	5	10.91	8.1	8.5	11.49	8.2	10.9	12.30	9.3	7.3
		6	11.02	8.7	7.0	11.23	8.3	8.7	12.00	8.4	6.6
		7	11.88	9.1	9.5	11.56	8.2	10.3	12.40	8.6	6.0
		8	11.56	8.6	10.0	10.82	8.0	9.8	12.30	8.2	6.4
Calcium adequate:											
6	(2)	11.42	8.3	9.9	11.23	7.9	10.5	12.35	8.9	6.9	
7	(2)	12.36	8.7	8.9	11.24	8.3	6.9	12.72	9.0	5.8	

<sup>1</sup> Killed for autopsy.

<sup>2</sup> Mean of the lot.

After the pigs had been on experiment 84 days and the blood calcium had dropped to its lowest level, the serum phosphatase was

also somewhat below the level of that in the serum of the pigs receiving an adequate amount of calcium. The average of the five calcium-deficient pigs was 3.9 units per 100 cc. of serum, while that of lots 6 and 7 receiving an adequate amount of calcium and showing no calcium-deficient symptoms was 7.5 units.

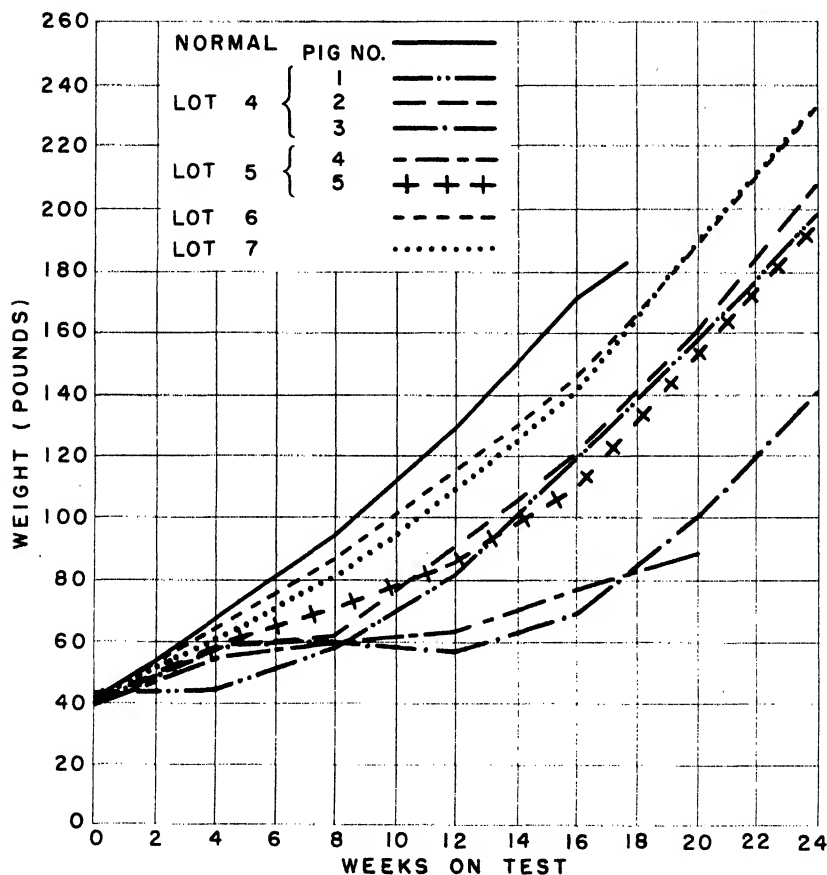


FIGURE 4.—Weights of the three pigs in lot 4, and pigs Nos. 4 and 5 of lot 5, showing calcium-deficiency symptoms, as compared with the weights of normal pigs, and with those of lots 6 and 7 which received adequate calcium. The calcium intakes of the animals in lots 4, 5, 6, and 7 were 0.10, 0.25, 0.41, and 0.55 percent, respectively. The curve for normal animals was drawn from data supplied by the Division of Animal Husbandry of the University of California and represents the average of the records of 457 pigs that had excellent rations (8).

#### [ EFFECT OF LOW-CALCIUM RATIONS ON DAILY GAINS AND BODY WEIGHT

One of the most interesting results of the feeding of low-calcium rations to young pigs was the effect produced on daily gain and body growth. The data for these two factors are recorded in table 4 and shown graphically in figure 4.

TABLE 4.—Daily gains and body weights of 5 pigs from lots 4 and 5, showing calcium-deficiency symptoms during experiment 2, and the means of these values for the pigs in lots 6 and 7, which received an adequate amount of calcium

[Weights recorded at 28-day intervals]

Lot No.	Pig No.	Initial body weight	28 days		56 days		84 days		112 days		140 days		168 days	
			Daily gain	Body weight	Daily gain	Body weight	Daily gain	Body weight	Daily gain	Body weight	Daily gain	Body weight	Daily gain	Body weight
Calcium deficient:		<i>Lb.</i>	<i>Lb.</i>	<i>Lb.</i>	<i>Lb.</i>	<i>Lb.</i>	<i>Lb.</i>	<i>Lb.</i>	<i>Lb.</i>	<i>Lb.</i>	<i>Lb.</i>	<i>Lb.</i>	<i>Lb.</i>	<i>Lb.</i>
4	1	43.3	0.02	44.0	0.46	57.0	0.85	81.0	1.35	119.0	1.35	157.0	1.46	198.0
	2	38.6	.65	57.0	.17	62.0	.96	89.0	1.10	120.0	1.42	160.0	1.71	208.0
	3	42.6	.55	58.0	.07	60.0	.15	56.0	.42	68.0	1.14	100.0	1.46	141.0
5	4	38.6	.55	54.0	.21	60.0	.10	63.0	.50	77.0	.39	88.0	( <sup>1</sup> )	
	5	41.6	.58	58.0	.42	70.0	.57	86.0	.85	110.0	1.53	153.0	1.50	195.0
Calcium adequate:														
6	( <sup>2</sup> )	41.4	.76	62.7	.85	86.7	.99	114.5	1.12	146.0	1.48	187.7	1.60	232.5
7	( <sup>2</sup> )	41.9	.62	59.5	.76	81.0	1.00	109.2	1.16	141.7	1.67	188.5	1.57	232.7

<sup>1</sup> Killed for autopsy.<sup>2</sup> Mean of lot.

The pigs in lot 4, and pigs Nos. 4 and 5, lot 5, which showed calcium-deficiency symptoms, made slow daily gains and poor body growth almost from the beginning of the experiment until the one hundred and twelfth day. After this period of slow growth daily gains materially improved so that during the latter part of the experiment their gains were almost as good as those of the pigs in lots 6 and 7, which received an adequate amount of calcium (table 4). The initial rate of gain for calcium-deficient pigs was quite different from that of normal pigs or that of the pigs in lots 6 and 7 which received an adequate amount of calcium, as is shown in figure 4.

The recovery in the rapidity of daily gain seems to have preceded the depression and recovery of the serum calcium values discussed previously. Thus it appears that pigs as they increase in age are able to recover from the effects of a limited calcium deficiency even though the level of calcium in the ration is unchanged. This would indicate that the requirements for calcium are greater in early life and become less as the pig matures. These findings support those of Mitchell and McClure (10) in respect to the calcium requirements for pigs.

#### SUMMARY

The results of an investigation to determine the effects of feeding calcium at different levels in the rations of 28 young pigs are reported.

Two experiments, one with three lots of pigs and one with four, were carried on for 24 weeks.

Data are presented to show (1) the effect of a deficiency of calcium in the ration on the growth and development of pigs, and (2) the minimum amount of calcium necessary in the ration for normal growth and development.

The results obtained indicate that the abnormalities resulting from feeding low-calcium rations are: (1) Anorexia; (2) an unthrifty appearance, characterized by emaciation and a rough coat of hair; (3) usually, but not always, unsteadiness in legs and finally posterior

paralysis; (4) a failure of normal growth and development of bone; and (5) poor utilization of feed.

Under the conditions of the experiments a level of 0.25 percent of calcium in a ration was found to be inadequate, whereas 0.41 percent was definitely adequate for the normal development of young pigs.

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# DROUGHT TOLERANCE IN SNAP BEANS<sup>1</sup>

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## INTRODUCTION

When the U. S. No. 1 Refugee bean was first tested at various places in the United States in 1934 it was reported to possess some tolerance to drought conditions. One report indicated that it had made a fair yield of snap beans while standard bean varieties in the same test did not produce a crop. Because of these reports, attempts were made to determine the degree to which U. S. No. 1 Refugee possessed the ability to yield well under adverse conditions of soil moisture, and whether strains related to it possessed more or less tolerance to the same conditions. The tests were conducted at the United States Horticultural Field Station at Cheyenne, Wyo.

## MATERIAL AND METHODS

The U. S. No. 1 Refugee bean was selected from the tenth generation of a cross of Stringless Green Refugee  $\times$  Wells Red Kidney. In developing this variety several other slightly different strains were carried along but not chosen for introduction. Some of these strains were used in this test. U. S. No. 1 Refugee is closely related to U. S. No. 5 Refugee, since the parents of the latter variety are U. S. No. 1 Refugee and a mosaic-resistant Refugee rogue (a strain of Corbett Refugee). U. S. No. 5 Refugee was released in 1935. All the strains with dashes separating parts of the numbers are from the  $F_3$  or later generations of U. S. No. 1 Refugee  $\times$  Refugee rogue and are essentially variable sister lines of U. S. No. 5 Refugee. It should be pointed out that these  $F_3$  lines were not fixed, and as they were increased there was undoubtedly differential survival of different types within the genetic complex. All other numbered strains are closely related to U. S. No. 1 Refugee. Five commercial varieties widely grown in the United States were carried for 4 years as controls, and in addition Burpee Stringless Greenpod, for 3 years.

In all years the varieties were planted in blocks planned for variance analysis. However, treatments and number of replications varied from year to year, as did somewhat the size of plot. The rows were  $3\frac{1}{2}$  feet apart, 50 feet long in 1935, and 25 feet long in the other 3 years. There were four plots of each strain in 1935, six in 1936, and eight in the last 2 years. In 1935 all plots were irrigated lightly once during the season. In 1936 and 1937 no irrigation was applied. In 1938 normally irrigated plots were randomized in with the unirrigated plots, to measure better the effect of drought conditions.

<sup>1</sup> Received for publication January 2, 1941.

All data are based on the accumulated yields per plot of snap beans (*Phaseolus vulgaris* L.) made on seven or eight picking dates of each season. Records were kept of the number of pods per pound at each picking.

### EFFECT OF DROUGHT ON YIELD

The yields of snap beans by varieties and strains, grown with and without irrigation, are shown in table 1.

TABLE 1.—Yield of snap beans by 35 varieties and strains in tests at Cheyenne, Wyo., 1935-38

Varieties and strains	Acre yield					
	1935 (1 light irrigation)	1936 (no irrigation)	1937 (no irrigation)	1938		Average for 3 years 1935-37
				No irri- gation	Normal irrigation	
	Pounds	Pounds	Pounds	Pounds	Pounds	Pounds
367	12,250	4,361	4,740			7,117
344	11,347	4,208	4,865			6,807
388-6-2	11,324	4,464	4,398	5,140	7,529	6,729
285	11,293	4,464	5,285	4,916	6,290	7,014
285	11,044	4,184	5,170			6,799
282	11,001	3,837	4,849			6,562
229	10,779	4,291	5,026			6,699
305	10,748	4,615	4,906	4,094	3,933	6,756
391-15-2	10,659	3,694	3,907	4,430	4,082	6,087
388-1-5	10,612	3,287	3,975	4,431	8,139	5,958
287	10,534	4,070	5,213	3,871	5,177	6,606
388-8-5	10,351	3,663	4,020			6,014
304	10,332	4,527	5,221	4,343	5,152	6,083
280	10,316	3,918	5,003			6,412
919	10,215	4,063	5,116	4,092	4,779	6,775
283	10,149	5,159	5,433			6,914
309	10,071	4,337	5,048	4,493	4,742	6,485
U. S. No. 5 Refugee	9,916	3,077	4,031	6,472	7,355	5,675
391-1-4	9,943	4,952	5,485	4,916	6,596	6,793
228	9,818	4,410	4,838	4,244	7,156	6,364
308	9,706	4,889	4,906			6,500
391-15-11	9,682	3,881	4,398			5,987
391-15-4	9,603	3,272	3,237			5,367
388-7-6	9,527	3,770	4,157			5,818
U. S. No. 1 Refugee	9,223	3,963	4,927	4,629	5,015	6,048
328	9,157	3,261	3,784			5,401
Stringless Green Refugee <sup>1</sup>	9,106	4,343	3,959	5,053	7,940	5,803
Giant Stringless Greenpod <sup>1</sup>	8,846	4,672	4,138	4,095	4,057	5,885
342	8,768	3,942	4,540			5,750
284	8,745	4,304	5,112	5,090	5,849	6,054
Full Measure <sup>1</sup>	8,589	3,612	3,343	4,406	3,783	5,181
341	8,367	3,230	4,455			5,351
Brittle Wax <sup>1</sup>	7,442	3,430	4,068	3,273	3,535	4,980
New Stringless Greenpod <sup>1</sup>	6,462	4,029	3,864	2,639	3,534	4,785
Burpee Stringless Greenpod <sup>1</sup>	5,948	4,571	4,122			4,880
Total	341,863	143,710	159,548	85,227	104,613	
Average	9,768	4,106	4,559	4,486	5,506	

<sup>1</sup> Commercial variety.

In 1935 the yields for an average of four plots varied from 12,250 pounds per acre for No. 367 (a U. S. No. 1 Refugee sib) to 5,948 pounds for Burpee Stringless Greenpod. A difference of 2,158 pounds was required for significance between strain means if odds of 19 to 1 are accepted as a criterion (table 1). In 1936 the yields for an average of six plots varied from 5,159 pounds for No. 283 (a U. S. No. 1 Refugee sib) to 3,077 pounds for U. S. No. 5 Refugee. A difference of 1,301 pounds was required for significance between the means.

In 1937 the yields varied from 5,485 pounds for No. 391-1-4 to 3,237 pounds for No. 391-15-4, with a difference of 412 pounds being required for significance.

In 1937 the plantings were so made that there were two series of plots to a block, and four blocks. The first of these blocks was approximately 40 feet from a windbreak to the west of the planting. Figure 1 shows the plots with block 1 in the foreground. The windbreak, consisting of a single row of poplars, is just out of the picture at the right.



FIGURE 1.—View of the field plots in 1937, with block 1 in the foreground. The western windbreak is just out of the picture at the right.

break made somewhat greater yields than those farther away. The method of planting adopted in 1937 made it possible to measure this effect of the windbreak. Thus, large variances were obtained for both blocks and series ( $F$  values exceeding the 1-percent point), and the average yield decreased as the distance from the windbreak increased. However, the relative ranking of the varieties and strains remained approximately the same regardless of proximity to the windbreak.

The average yield for the 35 varieties and strains in the block nearest the windbreak was 5,080 pounds, while the average for these same varieties and strains in the fourth block was 3,859 pounds. The averages for the second and third blocks were 4,758 and 4,526, respectively. Since only 135 pounds is required for significance in this case, all the differences between blocks are significant.

In 1938, seed of many of the strains was exhausted; so only 19 varieties and strains instead of 35 were carried. A comparison of the average yields of these 19 varieties and strains for the 4 years shows



that No. 286, a strain closely related to U. S. No. 1 Refugee, ranked first, and No. 388-6-2, a strain closely related to U. S. No. 5 Refugee, was second. Of the commercial varieties, Stringless Green Refugee ranked twelfth, Giant Stringless Greenpod sixteenth, Full Measure seventeenth, Brittle Wax eighteenth, and New Stringless Greenpod nineteenth. U. S. No. 5 Refugee ranked eleventh and U. S. No. 1 Refugee fourteenth.

If all 35 varieties and strains are included and ranked according to yield for the average of all plantings, it is found that strains 367, 283, 344, and 285 yielded significantly more than Stringless Green Refugee, the highest yielding commercial variety, which ranked twenty-second with a yield of 5,976 pounds. The other commercial varieties ranked as follows: Giant Stringless Greenpod, twenty-eighth; Full Measure, thirty-second; Burpee Stringless Greenpod, thirty-third; Brittle Wax, thirty-fourth; and New Stringless Greenpod, thirty-fifth; with acre yields of 5,433, 4,910, 4,880, 4,586, and 4,360 pounds, respectively. A significant difference for the comparison of mean yields for 4 years is 746 pounds, while if the 4-year averages are compared with the averages of the first 3 years a difference of 800 pounds is required.

No method of ranking based on either irrigated or nonirrigated plots or both will give a very high ranking to the commercial varieties. With the exception of Stringless Green Refugee, the commercial varieties are always near the bottom.

The results of irrigation in 1938 were complicated by the appearance of bacterial blight (*Bacterium phaseoli* E. F. Smith and *Bact. medicaginis* var. *phaseolicola* Burk.).<sup>2</sup> An effort was made to evaluate the influence of bacterial blight by estimating the amount of damage done on a scale of 0 to 5,<sup>3</sup> and at the end of the season the number of plants dead from blight was recorded. Adjustments on the basis of dead plants gave unreasonably high yields for the remaining plants, so further consideration was not given to adjusted yields. A variance analysis of the damage ratings was made in which the *F* values for irrigation and for varieties both exceeded the 1-percent point. Significant differences in disease evaluation between the mean of 80 irrigated and 80 nonirrigated plots was calculated to be 0.162. The actual mean difference is 0.344. The mean value of the irrigated plots was 3.888, while that for nonirrigated plots was 3.544.

Comparisons of blight damage within and between varieties are, however, more difficult, since the seed for most of the commercial varieties had been grown in relatively blight-free areas, while the seed of U. S. No. 1 Refugee and U. S. No. 5 Refugee and all other numbered strains had been grown at Greeley, Colo., where bacterial blight is fairly common every year. Four varieties showed a nonsignificant decrease in bacterial blight with irrigation, 12 varieties showed a nonsignificant increase, and only 4 showed a significant increase. In the nonirrigated beans, U. S. No. 5 Refugee and Full Measure both had the same low average value for bacterial blight (2.625 mean of 4 plots), and Stringless Green Refugee was not significantly different.

<sup>2</sup> Synonyms: *Phytophthora phaseoli* (E. F. Smith) Bergey et al., and *P. medicaginis* var. *phaseolica* Burkholder.

<sup>3</sup> 0=healthy; 5= very severe infection.

All other strains and varieties had significantly more blight damage than did the first two varieties. In the irrigated plots, U. S. No. 5 Refugee was low, with an average value of 3.125, while 10 strains showed significantly greater injury.

#### EFFECT OF DROUGHT ON SIZE OF POD

To make possible a study of the effects of drought on size of pod, counts were made at each harvest date of the number of pods per pound for each variety and strain.

The variations in number of pods per pound at the various harvest dates are shown graphically in figures 2, 3, 4, and 5 so that comparisons

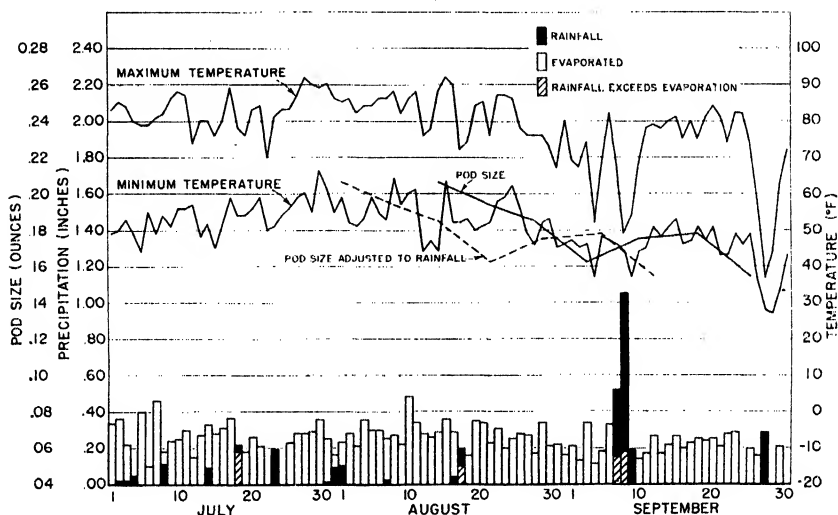


FIGURE 2.—Comparison of effects of maximum and minimum temperatures, evaporation, and precipitation on average pod size of 35 varieties and strains of snap beans, 1935.

may be made between them and such factors as precipitation, maximum and minimum temperatures, and evaporation. The average pod size in ounces is shown by a solid line, whereas a broken line represents pod size for dates 14 days prior to the actual dates of harvest. This adjusted line was set forward by a period of 14 days because an examination of the data showed that as a rule this was the length of time that usually elapsed between the occurrence of rainfall and response by the plants as measured by an increase in the size of the pods. In those instances where this rule holds true, the maximum and minimum points of the adjusted line should coincide with periods of rainfall and drought, respectively. In a few instances there appears to have been a period of only 6 or 7 days between rainfall and an increase in pod size. A more exact measurement of the time required for the plants to respond is not possible, since the harvests were made only at weekly intervals.

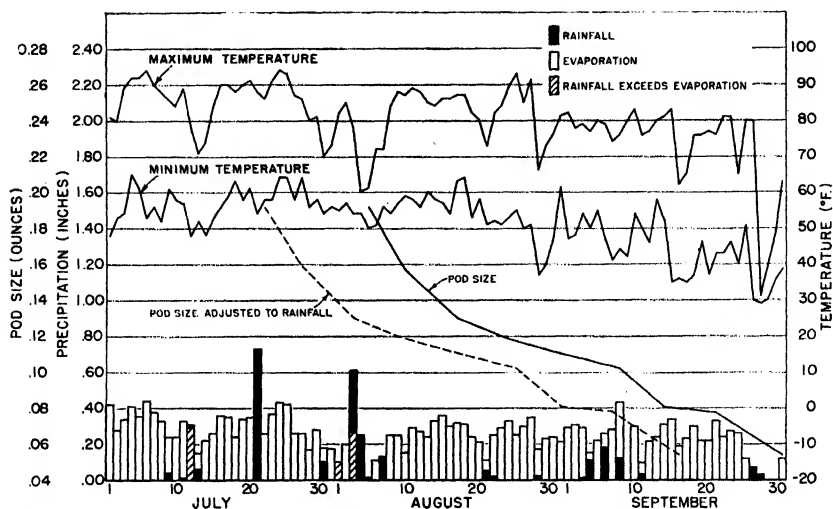


FIGURE 3.—Comparison of effects of maximum and minimum temperatures, evaporation, and precipitation on average pod size of 35 varieties and strains of snap beans, 1936.

Examination of figure 2, for 1935, appears at first glance to show but little correspondence between rainfall and pod size. However, a light irrigation given between the first and second harvest periods

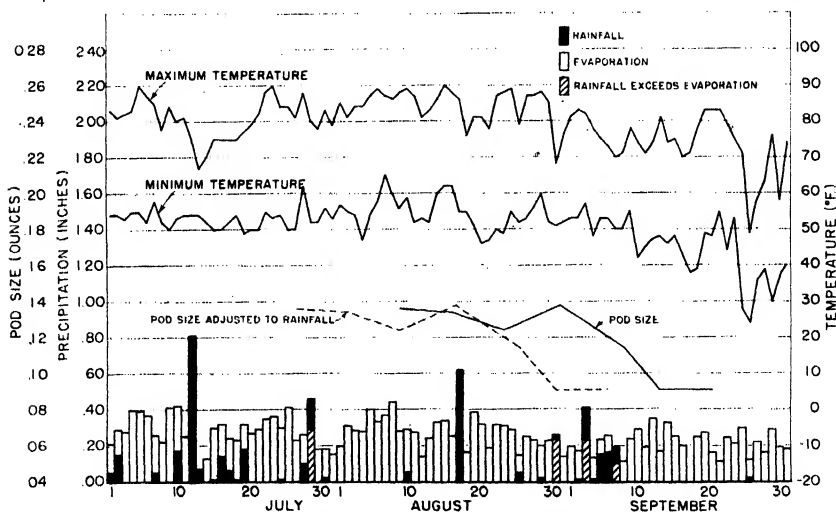


FIGURE 4.—Comparison of effects of maximum and minimum temperatures, evaporation, and precipitation on average pod size of 35 varieties and strains of snap beans, 1937.

was sufficient to cause a slight increase in pod size about 2 weeks later, and a rainy period from the 7th to the 9th of August further increased the upward trend. In this latter instance the lag in response of the plants did not exceed 10 days.

In 1936 (fig. 3) there was a rapid decrease in pod size from August 5, the date of the first harvest, to August 17, at which time the effects of the rainfall of August 3 and 4 became evident through a slight checking of the rapid decrease. From September 8 to 14 there was another period of rapid decrease in pod size, which was checked by the rains of September 4, 6, and 8. Thus, in the first instance there was a lag of approximately 14 days between the occurrence of rain and plant response as measured by a decrease in pod size, and in the second instance the effects of rain were evident in from 7 to 11 days.

In figure 4, for 1937, there is an almost perfect agreement between the adjusted line for pod size and the occurrence of rainfall. Thus,

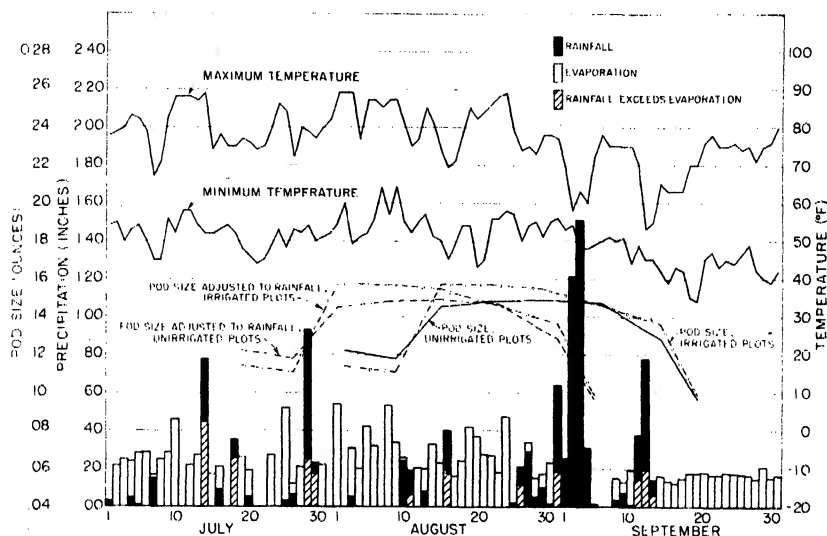


FIGURE 5.—Comparison of effects of maximum and minimum temperatures, evaporation, and precipitation on average pod size of 19 snap bean varieties grown under irrigation and under dry-land conditions, 1938.

the rainfall of August 17 had checked the rapid decrease in pod size by August 23, and by August 31 it had produced a measurable increase. Likewise, the rainfall of August 30 served to check a rapid decrease by September 13, and subsequent rains on September 3, 5, 6, and 7 prevented any further decrease to the end of the harvest season. From these data it appears that there was a lag of 7 to 14 days between a period of rainfall and response by the plants.

In figure 5, for 1938, it is possible to make a comparison of average pod size from irrigated and unirrigated plots. In this year, however, the rainfall of July, August, and September amounted to 9.74 inches, as compared with 3.21 inches in 1935, 2.97 inches in 1936, and 4.24 inches in 1937 (table 2). This amount of rainfall was evidently sufficient to promote almost normal growth, since additional water supplied through irrigation increased pod size to a measurable extent only at the third, fourth, and fifth harvest dates.

TABLE 2.—*Precipitation and evaporation at the United States Horticultural Field Station, Cheyenne, Wyo., 1935-38*

Month	Precipitation				Evaporation			
	1935	1936	1937	1938	1935	1936	1937	1938
	<i>Inches</i>	<i>Inches</i>	<i>Inches</i>	<i>Inches</i>	<i>Inches</i>	<i>Inches</i>	<i>Inches</i>	<i>Inches</i>
January.....	0.10	0.14	0.34	0.17				
February.....	.39	.34	.17	.10				
March.....	.09	.62	1.70	1.21				
April.....	2.78	2.05	1.23	2.41				
May.....	5.57	2.19	1.91	2.74		6.721	6.197	6.771
June.....	2.92	2.61	2.41	2.42	6.693	8.505	6.743	6.480
July.....	.80	1.25	2.29	2.76	7.578	6.363	8.603	6.959
August.....	.36	1.17	1.00	2.28	8.363	7.331	8.596	7.087
September.....	2.05	.55	.95	4.70	5.443	6.687	6.218	6.956
Total for July, August, and September.....	3.21	2.97	4.24	9.74	21.384	20.381	23.417	21.002
October.....	.25	.58	1.02	.52				
November.....	.19	.21	.20	.42				
December.....	.03	.36	.79	.51				
Total for the year.....	15.53	12.07	14.01	20.24				

A comparison of the line representing pod size in 1938 with those of the other 3 years shows that it was much more level during a major portion of the harvest season. Coupled with the fact that there was a much more even distribution of rainfall in 1938 than in any of the other 3 years, this may be taken as added evidence that rainfall was the most important factor of those measured in determining pod size.

From these data it is impossible to demonstrate whether temperatures directly influence pod size or whether they are merely concomitant phenomena. It is fairly evident that temperature extremes are not consistently related to pod size, and so it may be assumed that their departure from the optimum range was not sufficient to be an important determining factor. General temperature trends, on the other hand, show a striking resemblance to those for changes in pod size. To this rule, however, there are several important exceptions, and it is believed that the available moisture supply rather than temperature is the important deciding factor.

TABLE 3.—*Annual average number of pods of snap beans per pound produced in tests at Cheyenne, Wyo., 1935-38*

[I listed in order of average performance for all years under test]

Varieties and strains	Average number of pods per pound				
	1935 (1 light irrigation)	1936 (no irri- gation)	1937 (no irri- gation)	1938	
				No irri- gation	Normal irrigation
388-8-5.....	107.75	188.06	166.46		
367.....	103.67	172.83	159.92		
391-15-4.....	112.83	153.17	144.17		
344.....	96.00	154.78	149.08		
328.....	99.42	155.56	137.88		
391-15-2.....	99.17	145.94	146.38	131.42	130.83
280.....	90.25	160.67	140.46		
282.....	96.17	157.28	135.38		
306.....	100.50	154.28	128.67		
U. S. No. 5 Refugee.....	98.83	176.56	146.83	120.58	95.08

TABLE 3.—*Annual average number of pods of snap beans per pound produced in tests at Cheyenne, Wyo., 1935-38—Continued*

Varieties and strains	Average number of pods per pound				
	1935	1936	1937	1938	
	(1 light irrigation)	(no irrigation)	(no irrigation)	No irrigation	Normal irrigation
391-1-4	104.17	158.00	135.54	121.25	115.92
305	93.08	149.00	131.71	126.42	134.50
309	98.33	155.83	133.13	117.25	124.25
287	99.00	153.61	135.88	124.08	115.25
285	100.00	149.94	129.67		
U. S. No. 1 Refugee	96.08	150.22	129.04	123.25	125.17
388-1-5	83.17	176.44	146.04	108.58	106.17
283	94.42	147.89	129.88		
229	95.92	141.11	134.92		
286	93.00	151.72	131.54	121.50	122.08
304	95.75	148.61	131.75	123.25	116.75
284	98.83	149.67	132.04	117.67	116.25
Stringless Green Refugee	99.08	151.33	143.29	118.58	103.00
919	97.25	154.17	132.58	110.00	113.83
228	98.33	151.11	127.67	118.92	109.50
388-7-6	114.42	128.67	119.33		
391-15-11	89.75	133.72	127.13		
341	86.25	135.94	126.38		
388-6-2	87.00	131.99	121.58	120.00	110.75
342	83.00	130.89	125.54		
Brittle Wax	79.92	114.83	100.96	94.08	98.50
Giant Stringless Greenpod	74.67	114.44	103.50	99.17	91.67
Burpee Stringless Greenpod	86.33	106.89	94.04		
Full Measure	68.42	101.56	105.38	95.83	97.17
New Stringless Greenpod	66.67	95.00	104.42	103.33	92.33
Average for all varieties	93.93	145.76	131.05	115.53	111.53
Significant difference between varieties	11.71	14.45	9.91	15.49	15.49
Standard error	4.14	5.11	3.50	5.48	5.48

Table 3 shows as seasonal averages the number of pods per pound for the varieties and strains of beans tested each year. The order of listing these varieties and strains was determined by taking an average of their performances for all the years that they were under test. Thus, strain 388-8-5, which was under test for 3 years, produced on an average the greatest number of pods per pound, whereas New Stringless Greenpod produced the least number of pods per pound for the 4 years that it was under test.

Exact comparisons of the performances of these varieties and strains from year to year are not possible because of changes in experimental design that made a generalized analysis of the data for all years impossible. It is interesting to note, however, that the average number of pods per pound is greater for the dry seasons of 1936 and 1937 than it is for 1935, when one irrigation was given, or for 1938, the year of most abundant rainfall.

Comparisons between varieties and strains grown in any given year may be made with the aid of the values for significance appearing at the bottom of table 3. By such comparisons it is evident that in some varieties pod size is a fairly stable characteristic. This is especially true of the commercial varieties Brittle Wax, Giant Stringless Greenpod, Full Measure, New Stringless Greenpod, and Burpee Stringless Greenpod, which, with the exception of the last-named variety in 1935, consistently produced larger pods than most of the hybrid strains or than U. S. No. 1 Refugee or U. S. No. 5 Refugee.

On the other hand, such unstable strains as 344, 285, 280, 388-1-5, or 388-7-6 showed relatively much greater variability from year to year. Except in 1937, U. S. No. 1 Refugee did not differ significantly in pod size from Stringless Green Refugee; but U. S. No. 5 Refugee was more variable, as may be seen by its much greater reduction in size under conditions of extreme drought such as existed in 1936 and 1937.

As has already been stated, counts were made of the number of pods per pound at each harvest date; and though it did not seem worth while to analyze these data for each date, an analysis was made that included an early, a midseason, and a late harvest. This analysis showed that there was a highly significant interaction between varieties and periods for the years 1935, 1936, and 1937. In each case the *F* value exceeded the 1-percent point. In the interest of brevity, only the data for 1935 are shown in detail (table 4). By the aid of the values appended to table 4 it is possible to measure the significance of the various interactions and that of the differences between and within varieties at each harvest period.

TABLE 4.—Number of pods of snap beans per pound at early, midseason, and late harvest dates in tests at Cheyenne, Wyo., 1935

Varieties and strains	Early harvest	Mid-season harvest	Late harvest	Varieties and strains	Early harvest	Mid-season harvest	Late harvest
	<i>Number</i>	<i>Number</i>	<i>Number</i>		<i>Number</i>	<i>Number</i>	<i>Number</i>
388-8-5	101.50	112.75	109.00	284	74.00	115.00	107.50
387	103.25	109.25	98.50	Stringless Green Refugee	90.25	115.25	91.75
391-15-4	102.50	120.50	115.50	919	77.25	102.25	112.25
344	95.75	103.75	88.50	228	73.75	103.25	118.00
328	87.00	105.75	105.50	388-7-6	104.00	120.00	119.25
391-15-2	88.75	104.50	104.25	391-15-11	84.00	98.00	87.25
280	74.50	99.75	96.50	341	72.25	83.50	103.00
282	79.75	102.25	106.50	388-6-2	78.00	80.00	94.00
308	81.75	109.25	110.50	342	69.00	88.25	91.75
U. S. No. 5 Refugee	84.25	97.25	115.00	Brittle Wax	59.25	75.00	105.50
391-1-4	88.50	111.75	112.25	Giant Stringless Greenpod	59.75	77.75	86.50
305	78.50	98.00	102.75	Burpee Stringless Greenpod	65.25	73.75	120.00
309	77.00	93.50	124.50	Full Measure	58.00	66.50	80.75
287	82.25	111.75	103.00	New Stringless Greenpod	59.50	77.50	63.00
285	83.25	108.00	108.75				
U. S. No. 1 Refugee	84.00	106.00	68.25	Average	80.71	99.33	101.74
388-1-5	78.75	85.25	85.50				
283	80.75	98.25	104.25				
229	79.75	108.50	99.50				
286	82.50	100.50	96.00				
304	86.25	105.00	96.00				

Value for significance between averages for periods, 3.34; standard error, 1.18.

Value for significance between any two means in the table, 20.23; standard error, 7.17.

Value for significance for interaction "strains  $\times$  periods," 28.69.

From the data given it will be seen that 12 varieties and strains produced a greater number of pods per pound at the midseason than at the early harvest, and none produced less. Stringless Green Refugee shows a significant decrease in number of pods per pound between the midseason and late harvest periods, whereas No. 309, Brittle Wax, and Burpee Stringless Greenpod show an increase. Most of the interactions implied by these differences have been found to be significant.

## SUMMARY

During a 4-year period certain commercial varieties of snap beans were compared with U. S. No. 1 Refugee and U. S. No. 5 Refugee beans and several related strains to determine variations in their relative drought tolerance.

Under the conditions of these tests it was shown that U. S. No. 1 Refugee and U. S. No. 5 Refugee and certain hybrid strains possessed the ability to yield better under drought or semidrought conditions than the commercial varieties tested.

In 1938 the appearance of bacterial blight (*Bacterium phaseoli* E. F. Smith and *Bact. medicaginis* var. *phaseolicola* Burk.) complicated to some extent the determination of drought effects.

A study of the relative damage caused by bacterial blight on the 19 varieties and strains under test in the dry-land plots showed that U. S. No. 5 Refugee and Full Measure were the least injured, though Stringless Green Refugee was not significantly different. All other varieties and strains showed significantly greater damage than the first two varieties. In the irrigated plots U. S. No. 5 Refugee showed the least injury, and 10 varieties and strains were significantly more damaged.

A comparison of the effects of maximum and minimum temperatures, evaporation, and rainfall on pod size showed that rainfall was the most important determining factor. In general, there was a lapse of from 7 to 14 days from the time that rain fell until there was a measurable difference in pod size.

A majority of the varieties and strains under test showed a significant decrease in pod size whenever available soil moisture became a limiting factor. Certain of them, however, showed an actual increase in pod size.

In general, the commercial varieties were more stable with respect to pod size than the hybrid strains.





# MOTTLE LEAF, A VIRUS DISEASE OF CHERRIES<sup>1</sup>

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## INTRODUCTION

A graft-transmissible disease of cherry trees herein referred to as mottle leaf is known to have existed in different sections of the Pacific Northwest for the last several years. In 1917 D. F. Fisher photographed<sup>3</sup> cherry leaf specimens collected near Monitor, Wash., which exhibited symptoms similar to mottle leaf. Zeller (8)<sup>4</sup> reported having observed the disease in 1920 on Napoleon (Royal Ann) cherry trees growing in Oregon. Leaf specimens exhibiting typical mottle leaf symptoms were observed in the collection of Dr. C. W. Hungerford, University of Idaho, from Bing cherry trees growing in the Lewiston (Idaho) district, and collected September 1, 1922. Cherry leaves collected by B. F. Dana near Kennewick, Wash., during the summer of 1924 and deposited in the collections of the Department of Plant Pathology, State College of Washington, have also been examined, and these apparently represent typical mottle leaf. Several orchardists near Wenatchee, Wash., have given interesting accounts of the early appearance of peculiar malformed leaves on their cherry trees, and it is considered probable that as early as 1910 a few cherry trees growing in the north central part of Washington were affected by mottle leaf. At the present time sweet cherry trees growing in Washington, Oregon, Idaho, and British Columbia have been found to be affected by the disorder. In California, one Waterhouse cherry tree was observed that exhibited symptoms considered typical of mottle leaf.

Preliminary investigations reported in 1935 by McLarty<sup>5</sup> and by Reeves (6) demonstrated that mottle leaf was a graft-transmissible disease of sweet cherries. Previously, the disorder had been vaguely attributed to various factors such as unfavorable soil conditions, severe spring frosts, rosette or little leaf, poor root systems, chlorosis, and crown gall. Subsequent observations and transmission tests have definitely indicated that the disease is of virus origin and also have revealed that variations of symptom expression occur on different varieties of both sweet and sour cherries. Mottle leaf has been observed occurring naturally under field conditions on the sweet cherry (*Prunus avium* L.) and the wild cherry (*P. emarginata* (Dougl.) Walp.), but it has been transmitted to other species of *Prunus*. The

<sup>1</sup> Received for publication December 31, 1940. The material presented herein represents the substance of a thesis submitted to the faculty of the Graduate School of the State College of Washington in partial fulfillment of the requirements for the degree of master of science.

<sup>2</sup> Special appreciation is expressed to C. P. Harley, of this Division, and to Dr. F. D. Heald, of the State College of Washington, for advice and suggestions during the course of this work; and to Dr. Lee M. Hutchins and Dr. John W. Roberts, of this Division, for helpful criticism of the manuscript.

<sup>3</sup> Unpublished photograph is in possession of author.

<sup>4</sup> Italic numbers in parentheses refer to Literature Cited, p. 571.

<sup>5</sup> McLARTY, H. R. CHERRY MOTTLE LEAF. Northwest Assoc. Hort., Ent. and Plant Path., Abs. Papers 1: 5. 1935. [Mimeographed.]

purpose of this paper is to present the results of studies on symptoms and transmission of cherry mottle leaf, together with incidental data gathered during the course of the investigation.

### LEAF SYMPTOMS

The leaves of cherry trees affected by mottle leaf exhibit the most distinctive symptoms of the disorder, although a considerable variation in the intensity of the leaf symptoms exists among different cherry varieties and to some extent among different trees of the same variety. In the Wenatchee, Wash., district, where most of the observations have been made, the most commonly affected variety is the Bing. This variety is important commercially in the Pacific Northwest, particularly in the State of Washington, where Bing constitutes approximately 58 percent of all sweet cherry trees according to a tree survey made in 1936.<sup>6</sup>

The leaves from a severely affected Bing tree shown in figure 1, *A*, exhibit the mottling of color and puckered leaf condition characteristic of the disorder. The periphery of the leaves is irregular, and midvein and lateral veins failed to develop as in a normal leaf, with the result that the leaf has become puckered and misshapen. Light-yellow or yellowish-green areas and spots appear in the interveinal tissue and along the veins. These chlorotic areas become apparent soon after the leaves unfold in the spring, and produce the conspicuous mottling that on the Bing variety remains evident throughout the period of foliation. No retarded foliation of diseased trees has been observed, and the leaves do not fall prematurely. Some of the spots and irregular light-colored areas may drop out, leaving holes or irregular perforations in the leaf. The chlorotic areas are quite translucent in transmitted light, in contrast to the nearly opaque green tissue of the leaf. Practically all leaves on a severely diseased Bing tree exhibit symptoms and on the average are smaller than the leaves of a healthy tree. Figure 1, *B*, illustrates an extreme difference in size between leaves from a healthy tree and leaves from a severely diseased tree, but the average difference is striking enough to be noticeable. When one becomes familiar with mottle leaf symptoms, affected trees may be recognized from a considerable distance because of the abnormal appearance of the foliage.

Certain variations in leaf symptoms may occur, particularly during the first year that a tree is naturally affected by mottle leaf. Examples of such variations are shown in figure 2. In *A* the leaf shows some ruffling and little mottling of color. In *B* and *C* the leaves exhibit no rugosity, but the chlorotic areas are plainly evident, approximating a veinlet clearing in *B* to a distinct chlorosis along the veinlets in *C*. In *D* the leaf is almost entirely chlorotic in appearance and slightly ruffled. The leaves of an average diseased Bing tree usually exhibit symptoms ranging from moderate (fig. 2, *E*) to the more intense or severe symptoms shown in figure 1, *A*. In figure 2, *F*, the leaf is from a healthy Bing tree. All the leaves in figure 2 showing symptoms were taken from naturally infected trees. Leaves with symptoms considered typical of mottle leaf are usually in greater abundance on a tree than those with atypical symptoms. In instances where

<sup>6</sup> HEISIG, C. P. WASHINGTON TREE FRUIT AND BERRY ACREAGE SURVEY. Wash. Agr. Expt. Sta. 1937. Mimeographed.]

diseased trees exhibit leaf symptoms similar to those in figure 2, *C* and *D*, the question might be raised as to whether some virus other than the infective principle responsible for typical mottle leaf



FIGURE 1.—Leaves from naturally infected Bing cherry trees, showing severe mottle leaf symptoms. *A*, Four leaves exhibiting the mottling of color and puckering characteristic of the disorder. About four-fifths natural size. (Photographed by transmitted light.) *B*, Twigs from a healthy (*a*) and from a diseased (*b*) Bing cherry tree, showing the difference in leaf size. About one-third natural size. (Photographed by transmitted and reflected light.)

might be responsible for the variable symptoms. This question cannot be definitely answered, although results of transmission tests up to the present time suggest that only one virus is involved, since

variable leaf symptoms have not appeared on graft-inoculated trees.

The first year a tree is affected by mottle leaf, the symptoms frequently appear only on leaves of a few branches. Usually in the

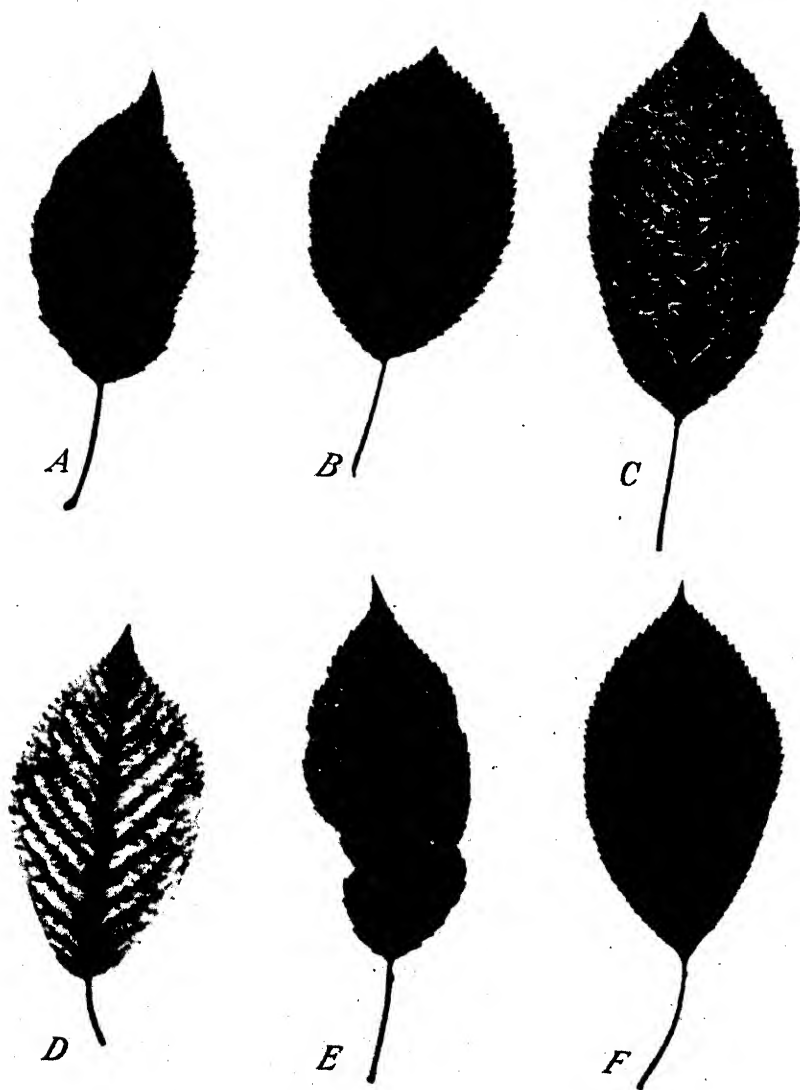


FIGURE 2.—A-D, Symptom variations of cherry mottle leaf; E, representative of mottle leaf symptoms of moderate intensity; F, leaf from a healthy tree. All leaves from Bing trees. See text for discussion. (Photographed by transmitted light.)

second year the majority of leaves are mottled and the symptoms become more pronounced; the leaves often decrease in size during succeeding years.

A few trees have been observed in which leaf symptoms were noticeably less intense than in preceding years. This decrease cannot be accounted for except, perhaps, on the basis of more favorable cultural and growing conditions. No tree has been known to recover from mottle leaf, and any decrease in severity of symptoms has not been sufficient to cause doubt as to the identity of the disorder.

#### EFFECT OF MOTTLE LEAF ON GROWTH AND FRUIT OF TREE

Studies on the effect of mottle leaf on the growth and fruit of the tree have been directed mainly to the Bing and Napoleon varieties. In general, the extent to which growth and fruit of the tree were affected could be correlated with the severity of leaf symptoms, the length of time the tree had been diseased, and the size of the fruit crop.

The terminal growth of diseased trees was less than that of healthy trees growing under similar cultural conditions. The first year a tree was affected there was little or no effect on the growth. After 2 or more years the reduction in terminal growth was especially noticeable if the leaf symptoms were severe. In advanced stages of the disease, the spurs made short growths and there was a general stunting of the tree.

Blossoming dates and the character of the blossoms have apparently not been influenced by mottle leaf, and there was little or no difference in the quality of the fruit the first year a tree exhibited leaf symptoms. Trees bearing a heavy crop of fruit and known to be severely affected by mottle leaf for 3 or more years have been found to produce but little marketable fruit, or none at all. The fruit on such trees was usually undersized, insipid, and late in ripening. After 2 or 3 years the disease evidently influences leaf function to the extent that a heavy crop of fruit cannot mature properly. When bearing only a light crop, a tree affected by mottle leaf may produce fruits of nearly normal size that are only slightly insipid. In general, however, Bing and Napoleon trees affected by mottle leaf for more than 2 years have not produced a commercial crop of fruit. Occasionally small, irregular, light-brown necrotic areas developed on the fruits of severely diseased Napoleon trees 3 or 4 weeks before harvest. As the fruit approached maturity the necrotic areas became slightly depressed, causing the fruit to become misshapen. These necrotic areas do not necessarily develop on the fruits of an affected tree in successive years, and factors other than the disease may be partly or wholly responsible for the condition.

#### VARIETAL SUSCEPTIBILITY

##### OCCURRENCE OF MOTTLE LEAF UNDER FIELD CONDITIONS

Under field conditions in the Pacific Northwest, only four varieties of sweet cherries (*Prunus avium* L.) have been found with mottle leaf symptoms. The Bing is most often affected, and because this variety exhibits definite and severe leaf symptoms it was selected as the test variety for experimental work. Naturally affected trees of the Napoleon (Royal Ann), a variety that constitutes approximately 17 percent <sup>7</sup> of the commercially planted sweet cherry trees in Washing-

<sup>7</sup> See footnote 6.

ton, often show leaf symptoms equally as severe as are shown by trees of the Bing variety. The Waterhouse or Long Stem Waterhouse, a rather unimportant variety commercially, also exhibits definite leaf symptoms. The fourth variety found affected under field conditions is the Lambert, which constitutes approximately 18 percent<sup>7</sup> of the commercially planted sweet cherries in Washington. However, only three Lambert trees with mottle leaf symptoms, all located in one orchard near Wenatchee, have been discovered. A comparatively few leaves on each tree were affected, and the symptoms were attenuated and somewhat fugitive. Moderate interveinal chlorosis, accompanied by a slight leaf ruffling (fig. 3), was found on

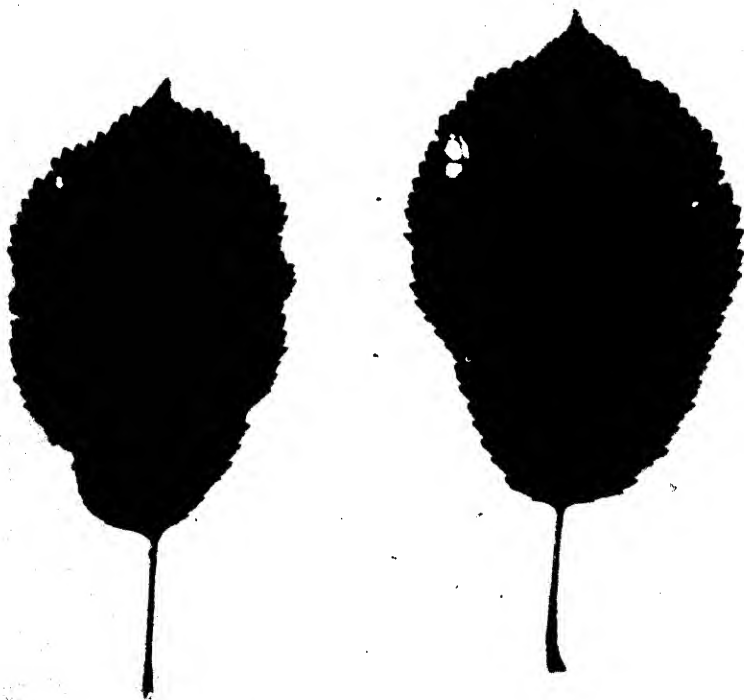


FIGURE 3.—Symptoms of mottle leaf in Lambert cherry, showing moderate interveinal chlorosis and slight leaf ruffling. (Photographed by transmitted and reflected light.)

about 20 percent of the leaves 5 to 6 weeks after growth started in the spring. The chlorosis was evident until shortly after harvest and then gradually disappeared, although the leaf ruffling persisted for some time. With these exceptions Lambert was found to be quite free from symptom expression.

#### TRANSMISSION EXPERIMENTS

In order to determine leaf symptoms of mottle leaf on varieties apparently free from the condition in the field, two methods of procedure

<sup>7</sup>See footnote 6.

were employed: (1) Buds of different cherry varieties were budded into diseased Bing trees, and (2) buds from diseased Bing trees were budded into healthy young trees of different varieties. In instances where leaf symptoms were not clearly discernible on the test trees infected by budding, buds were taken from such trees and placed in healthy young Bing trees to prove the presence of the virus in tissues of the variety so tested. No variety proved to be immune, although several varieties were highly tolerant and practically symptomless.

Eleven varieties of sweet cherries were tested by budding into diseased Bing trees, with the result that four varieties—Bing, Napoleon, Waterhouse, and Centennial—exhibited severe leaf symptoms. Somewhat variable symptom expression was obtained for Black Tartarian, Republican, Early Purple, and Governor Wood varieties, but the leaves were definitely ruffled and in a few instances chlorotic spots appeared. However, the mottling of these four varieties was mostly fugitive and was entirely absent from many trees. The leaf symptoms for the Deacon and Parkhill Seedling varieties proved to be much the same as for the Lambert described above.

Three varieties of sour cherries (*Prunus cerasus* L.) were tested and all proved tolerant to mottle leaf. The Montmorency and Early Richmond varieties exhibited occasional chlorotic spots on a few leaves, but no leaf ruffling was observed. No symptoms of any kind were found on leaves of English Morello.

Three varieties of duke cherries (*Prunus arium*  $\times$  *P. cerasus*) were tested and are considered tolerant to mottle leaf. Occasional leaves of the May Duke variety exhibited light mottling for a short time and were also slightly ruffled. Practically no leaf mottling was observed on either Late Duke or McKee, and there was only occasional slight ruffling of a few leaves.

Mahaleb trees (*Prunus mahaleb* L.) grown from seed and inoculated with mottle leaf by budding showed practically no leaf symptoms. However, when buds from healthy mahaleb trees were budded into diseased Bing trees, a few chlorotic spots and slight leaf curling developed on about 10 percent of the leaves on the growth from the mahaleb buds. The mahaleb was therefore considered partially tolerant to mottle leaf.

Wild cherry trees (*Prunus emarginata*) growing in their native habitat and budded with diseased Bing buds produced leaves with variable mottling and light leaf ruffling. When buds were taken from the wild cherry and budded into diseased Bing trees, a few of the wild cherry leaves showed light ruffling and a change of color approximating interveinal chlorosis. In 1936 leaves of somewhat similar appearance were observed on wild cherry trees growing under natural conditions (fig. 4). When buds taken from these trees were inserted into healthy Bing trees severe mottling characteristic of mottle leaf resulted. Some of the wild cherry trees on which the disease occurred naturally were growing within 40 feet of a mottled Bing tree. This would suggest a possible relationship between the disease on the Bing and that on the wild cherry because of their proximity. This particular situation indicates the possibility of an insect vector attacking both the sweet cherry and the native wild cherry, which may have an important bearing on the natural spread of the disease. In the north central part of Washington, it has been



commonly observed that trees affected with mottle leaf are more prevalent in orchards planted in canyons and in foothill orchards near which native wild cherry trees are growing.

Table 1 presents a summary of the leaf symptoms of mottle leaf exhibited by different species and varieties of cherries, with remarks concerning the relative intensity of symptom manifestation in the leaves. Since observations for some varieties were made on young trees and since leaf symptoms for a variety may be variable, this table will be subject to additions and revision as additional information is obtained.



FIGURE 4.—Symptoms of mottle leaf in a naturally infected native wild cherry (*Prunus emarginata*). Normal leaf at left. (Photographed by transmitted light.) Healthy Bing trees inoculated by budding from such wild cherry trees produced leaves with very severe mottle leaf symptoms similar to those illustrated in figure 1, B.

Attempts were made to transmit mottle leaf to different varieties of plum, peach, and apricot. Buds from diseased Bing trees were inserted into Climax plum, Italian Prune plum, Double  $\times$  Agen (French Petite) plum, Late Crawford peach, Triumph peach, Tilton apricot, Wenatchee Moorpark apricot, and Riland apricot. Five young trees of each variety were used in the experiment; two infective buds were inserted into each tree in 1936, and three or more buds in 1937. None of the buds lived, and upon examination no evidence was found of tissue union. Since no tissue union was established between diseased and healthy tissue, all trees remained free from mottle leaf. These tests, therefore, failed to show whether or not plums, peaches, and apricots are susceptible.

In 1939, under greenhouse conditions, healthy Bing scions were successfully grafted on branches of three J. H. Hale peach trees. Buds from diseased Bing trees were then inserted into trunks of the peach trees, and growth union was established. Typical mottle leaf

symptoms appeared on leaves of the Bing scions, which demonstrated that the virus had passed through the tissues of the peach. A few of the peach leaves also exhibited a slight mottling.

TABLE 1.—*Relative intensity of mottle leaf symptoms in species and varieties of cherries*

Species of cherry	Variety	Leaf symptoms	
		Mottling	Rugosity
<i>Prunus avium</i>	Bing	Pronounced	Severely puckered.
	Napoleon	do	Do.
	Waterhouse	Moderate	Puckered.
	Centennial	do	Do.
	Black Tartarian	Slight, variable	Moderately ruffled.
	Early Purple	do	Do.
	Governor Wood	do	Do.
	Republican	do	Do.
	Lambert	Slight, fugitive	Lightly ruffled.
	Deacon	do	Do.
	Parkhill seedling	do	Do.
<i>P. cerasus</i>	Montmorency	Occasional slight	None.
	Early Richmond	do	Do.
	English Morello	None	Do.
<i>P. avium</i> × <i>P. cerasus</i>	May Duke	Occasional, slight	Lightly ruffled.
	Late Duke	Very slight, fugitive	Occasional light ruffling.
<i>P. mahaleb</i>	McKee	do	Do.
	Mahaleb	Occasional, slight	Do.
<i>P. emarginata</i>	Wild cherry	Medium, fugitive	Do.

As previously mentioned, preliminary transmission tests, started in 1934 and reported in 1935 (6), indicated that mottle leaf could be transmitted from diseased to healthy cherry trees by the ordinary methods of budding or grafting; and further tests have demonstrated that the disease could be transmitted by any means where growth union was established between diseased and healthy tissues. In August 1935, buds from diseased trees were budded into 27 healthy trees of 9 different varieties of sweet and sour cherries. The infective buds remained in good condition until late in October 1935, when early cold weather killed all the buds and seriously injured most of the trees. With the exception of one tree, which was winter-killed, all trees later proved to be infected with mottle leaf. Later tests showed that any "patch-bark" graft was effective in transmitting the disease whenever growth union of tissues was established.

The time necessary for the development of leaf symptoms after artificial bud or graft inoculation apparently varies with different conditions. A scion from a diseased Bing tree was tongue-grafted on a small branch of a young healthy Napoleon tree March 28, 1935, before growth started. The first leaves coming out on the scion showed typical mottle leaf symptoms. On June 20, 1935, 84 days after the graft was made, the terminal leaves on an adjacent Napoleon shoot exhibited symptoms of mottle leaf (fig. 5). Later in the season mottle leaf also appeared on leaves of the current season's growth on other branches of the main lateral limb on which the inoculating scion had been grafted. Leaf symptoms did not appear on the leaves of other main lateral limbs until 1936.

In an experiment designed to test transmission of mottle leaf through the roots, an old diseased tree was cut down and roots  $\frac{1}{2}$  to  $1\frac{1}{2}$  inches in diameter were uncovered. On April 28, 1939, 20 young

healthy Bing trees were root-grafted to the roots of the diseased tree, and 7 showed definite leaf symptoms 60 days after the grafts were made. Later, all the trees that formed a growth union with the roots of the diseased tree showed mottle leaf symptoms.

In the following experiment, symptoms were obtained 37 days after infective buds were placed in healthy trees. Bud sticks taken from a diseased Bing tree in January 1937 were held in 32° F. storage until



FIGURE 5.—Spread of mottle leaf on a Napoleon cherry tree. On March 28, 1935, a scion from a diseased Bing tree was tongue-grafted on the small upper branch at the point indicated by arrow. All leaves on the scion show symptoms. A leaf on the lower branch, the tip of which is held by hand, was the first leaf of the newly infected tree to show symptoms. (Photographed July 2, 1935.)

June 5, 1937, and at this time the buds were inserted into healthy young Bing trees. On July 12, 1937, mottle leaf symptoms were observed on leaves of two of the seven trees used in the test. Under greenhouse conditions the time necessary for the development of leaf symptoms after bud inoculation was considerably shortened. When healthy young Bing trees growing in the greenhouse were so inoculated, faint leaf symptoms occurred on a few leaves in 9 days, and the same leaves showed definite mottle leaf 14 days after budding. Under field conditions, budding or grafting in midsummer or later failed to produce symptoms of the disease until the following spring.

Evidence that the infective principle of mottle leaf is transported in the tree by conductive tissues of the phloem was obtained by ringing experiments. A ring of bark about 4 to 6 mm. in width was removed from the trunk of each of 10 young Bing trees, from 3 to 4 feet in height, and diseased buds were inserted above or below the ring. Mottle leaf symptoms developed on the side of the ring where the infective bud was inserted, but in no instance did symptoms develop on the opposite side of the ring.

All mechanical methods of transmission employed, where no growth union of tissue was established, have been unsuccessful in transmitting mottle leaf. Many mechanical methods of transmitting virus diseases as used by other investigators, or adaptations thereof, have been employed in an attempt to transmit mottle leaf. Juice from diseased plants was obtained by grinding either leaves or tender stem tissues or both in a mortar and pressing the juice through cheesecloth. In some instances a small amount of distilled water was added during the grinding process. The juice thus obtained was used in different ways in an attempt to inoculate healthy cherry trees. The four principal methods employed were (1) injection of juice into the base of leaf petioles and tender stem tissue by means of a fine hypodermic needle; (2) the use of carborundum in applying juice to leaves as employed by Rawlins and Tompkins (5) except that a 400-mesh instead of a 600-mesh grade carborundum was employed; (3) rubbing leaves with a piece of cheesecloth saturated with juice without employing carborundum; and (4) placing a drop of juice on the leaf to be inoculated and pricking through the drop with a fine needle. Other methods employed were (1) binding together diseased and healthy leaves and pricking through them with fine needles; (2) holding a diseased leaf against the stem of a healthy plant and pricking the leaf with fine needles; and (3) cutting into stem tissue of a healthy plant with a knife used to cut stems or leaves of a diseased tree. No indication was obtained that transmission of mottle leaf was effected by any of the mechanical methods employed.

#### SPREAD OF MOTTLE LEAF IN THE FIELD

The manner in which mottle leaf of cherries spreads in the field is as yet unknown. It is considered probable, however, that an insect vector is responsible for natural mottle leaf infections. Only one insect, the black cherry aphid (*Myzus cerasi* (F.)), was tested as a possible vector in the present study. No evidence of transmission was obtained.

There was considerable variation in the rapidity with which the disease spread in different orchards under observation in the north-central part of Washington. In several orchards no new infections have occurred during the past 4 years, but, on the other hand, new infections have increased rapidly in a few. For example, in one small planting of 84 cherry trees, 6 showed mottle leaf in 1935; 1 additional tree developed leaf symptoms in 1936; no new infections occurred in 1937; then 21 additional trees showed leaf symptoms in 1938. Out of 52 cherry orchards in the north-central part of Washington, in which one or more diseased trees were located, the spread of the disorder has been considered serious in only 7 during the last 4 years.

### ATTEMPTS TO INACTIVATE THE VIRUS BY HOT-WATER TREATMENT

Following essentially the method employed by Kunkel (2), bud sticks from mottle leaf trees were subjected to hot-water treatments in an attempt to inactivate the virus. Bud sticks about 6 inches long and three-sixteenths to one-fourth of an inch in diameter were collected in January 1937 and held in cold storage until treated in April. Immediately after treatment the buds were inserted into healthy young cherry trees, and a scion with two buds was also tongue-grafted on each tree. The bud sticks were immersed in water held at the indicated temperatures for the following periods: (1) 41° C. for 15-, 60-, and 180-minute periods; (2) 46° for 10-, 30-, and 60-minute periods; and (3) 49° for a 10-minute period. Transmission of mottle leaf was obtained in all tests except by the buds treated at 41° for 180 minutes, in which instance no tissue union was established between the infective buds and the healthy trees. The buds subjected to 46° for 60 minutes and at 49° for 10 minutes were seriously weakened and ultimately died, although most of them lived about 3 weeks after budding, during which time tissue union was established. The experiment indicated that treatments severe enough to inactivate the virus would also kill the buds; however, a more extended range of temperature and treatment periods should be employed to definitely determine this. It is also recognized that different results might be obtained from similar experiments with budwood taken in July or August during the growing season, or perhaps a dry-heat treatment might be used that would inactivate the virus but would not kill the treated budwood.

### CHERRY DISORDERS SOMETIMES CONFUSED WITH MOTTLE LEAF

Certain disorders of cherry trees that cause leaf malformations have sometimes been confused with mottle leaf. For the purpose of comparing them with mottle leaf, brief mention will be made of those most commonly found in Washington cherry orchards.

Malformed leaves typical of those found on "unproductive-type" cherry trees in California (1), the condition sometimes referred to as "crinkle," have been observed on several trees in Washington and Idaho. Most of the affected trees in Washington had malformed leaves on only a few branches, although an occasional tree was found with practically all leaves more or less affected. Symptoms of the condition have been described by Kinman (1) and will not be further discussed here. Figure 6 shows typical malformed leaves from a Bing tree that differ considerably in appearance from leaves of a tree affected by mottle leaf.

Another disorder of cherry trees exhibiting a definite leaf symptom but differing from mottle leaf was seen on a few Bing trees near Wenatchee, Wash. (7). Leaf symptoms, which do not become evident until after the leaves are fully expanded, first appear as faint chlorotic spots or areas and rapidly become more definitely yellow (fig. 7). The most seriously mottled leaves soon take on late-season colors, form abscission layers, and shed. This leaf fall occurs 2 or 3 weeks prior to harvest, affecting from 30 to 50 percent of the leaves. This does not take place in trees affected by mottle leaf. After leaf fall

the remaining foliage appears somewhat wilted and the leaf spots are often edged by darker yellowish-brown areas, producing a somewhat rusty appearance. This disease, referred to in field notes as "rusty mottle" to distinguish it from other disorders, has been repeatedly transmitted to healthy trees by budding. Affected trees apparently have normal blossoms, and the fruit is normal in shape although its flavor is insipid. The growth of affected trees was fair but slightly less than that of comparable healthy trees.

Another unnamed disorder was found in 1938 on Bing and Lambert cherry trees in three different orchards in the Wenatchee district. The symptoms exhibited by the leaves were considered different from those of other cherry disorders causing leaf malformations. The leaves were characteristically elongate (fig. 8), with the lateral veins



FIGURE 6.—Four malformed leaves from an unproductive type of Bing cherry tree. This leaf deformity is sometimes referred to as "crinkle." (Photographed by transmitted light.)

not fully developed on one side, and often both sides, of the midvein. The periphery of some leaves was quite irregular and perforations occurred on a few. A faint interveinal chlorosis was present on some leaves during the late spring and early summer, but was not so evident later in the season. The surface of affected leaves was quite rough or ruffled, but not puckered as in mottle leaf. Usually from 25 to 50 percent of the leaves on a tree were malformed and the remainder were normal or nearly so. The symptoms somewhat resemble those of the condition referred to as "crinkle," but differ principally in that the affected leaves do not have the pronounced chlorotic areas near the leaf margin, are more elongated, and have a rough surface (fig. 8). The fruit was definitely affected on some of the trees, but little on

others. The fruits usually had a pronounced depression on the suture side, and for that reason the disorder has been referred to as "deep suture." No information has yet been obtained regarding the transmission of the disorder.



FIGURE 7.—Leaf symptoms of a naturally infected Bing cherry tree caused by an unnamed graft-transmissible disease, tentatively referred to as "rusty mottle." This disorder is considered different from mottle leaf. (Photographed by transmitted light.)

Another apparently different disorder on Bing, Napoleon, and Deacon varieties was found in four orchards in the Wenatchee district. The condition has been referred to in field notes as "lace leaf," because of the unusual number of leaf perforations that develop and

take on the general appearance of pieces of lace. No information has been obtained concerning the possible causes of the condition, although it is apparently not associated with unusual cultural or growing conditions.

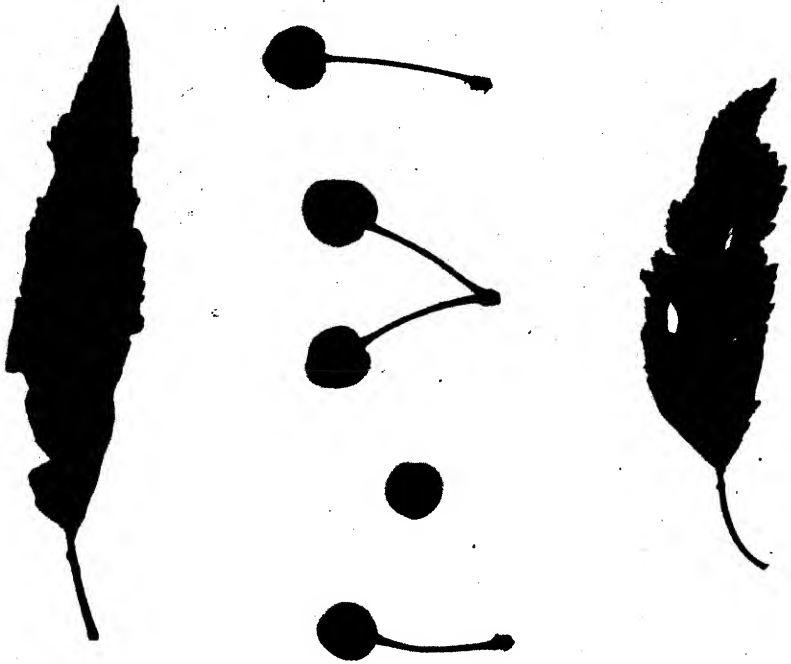


FIGURE 8.—Leaf and fruit symptoms on a Bing cherry tree caused by an unnamed disorder. Note the short lateral veins and unusual length of the leaves. Approximately one-half natural size. (Photographed by transmitted light.)

#### DISCUSSION

As was pointed out in the description of symptoms, there is considerable variability in the severity of the mottle leaf disease as indicated by the intensity of leaf symptoms during the first year a tree becomes affected. Healthy young Bing trees inoculated by buds or grafts exhibit approximately the same severity of leaf symptoms whether the infective tissue originated from a tree that had shown mottle leaf for many years or from one that had shown less severe symptoms for only 1 year. However, when the infective tissue originated from a naturally infected wild cherry tree, the leaf symptoms of inoculated young Bing trees were particularly severe, the resulting new shoots being quite small, severely mottled, and puckered. The growth of the young trees inoculated with the wild cherry mottle leaf were quite stunted. Observation of the inoculated trees for 2 years indicated that the disorder affecting the wild cherry was not only mottle



leaf, but a virulent form of the disease as judged by leaf symptoms of the inoculated trees. Whether the apparent virulence of mottle leaf can be increased by inoculating wild cherry trees with the disease from a Bing tree, and then inoculating a healthy Bing from the infected wild cherry tree has not yet been determined.

The presence of naturally infected wild cherry trees growing relatively near diseased Bing trees gave no indication as to which species first became infected. During 1937 and 1938 a large number of wild cherry trees growing in locations far removed from cultivated orchards were inspected for mottle leaf. As a result of this survey, wild cherry trees were found in two isolated localities with leaves having symptoms closely resembling those from trees proved to be infected by mottle leaf. Transmission tests completed in 1939 proved the wild cherry trees from one isolated locality to be infected by mottle leaf; tests are incomplete for the other group of trees. This may be considered as evidence to support the supposition that mottle leaf is indigenous to wild cherries in the north central part of Washington.

### CONTROL

The question of possible control measures is of particular interest to orchardists of the north central part of Washington who have cherry trees affected by mottle leaf. It has already been suggested (6) that diseased trees be taken out wherever possible, especially if they are affected to the extent that a profitable crop is not being produced. In discussing control measures, it might be well to point out that a tree once affected by mottle leaf has never been known to recover. Diseased trees cannot be cured by any known treatment, and removal of affected trees to prevent spread to healthy trees is the only suggested method of control. Because diseased trees usually produce good crops for 1 year, and often 2 years, after leaf symptoms are first evident, most growers hesitate to remove them. In most orchards under observation in the north central part of Washington mottle leaf spread very slowly during the years 1934 to 1938, inclusive. In a few orchards it has spread rapidly, however, and the presence of diseased trees is considered a menace to the remaining healthy trees in the orchard. In view of present knowledge of the disease the following suggestions for its control can be made: (1) Remove all trees that have shown leaf symptoms for 2 years or more in orchards where there has been little or no recent spread of mottle leaf; and (2) immediately remove all trees showing symptoms of mottle leaf in orchards where recent spread indicates that such trees are a menace to the remaining healthy trees in the orchard.

In instances where diseased trees have been removed, young trees have been reset in approximately the same location and have remained healthy for the four growing seasons during which they have been under observation.

It has been demonstrated (3, 4) that rootstocks influence the susceptibility of sweet cherry to buckskin, a graft-infectious disease of sweet cherry occurring in California. There is no evidence that the susceptibility of cherries to mottle leaf can be influenced by rootstocks except the observation that naturally infected Bing trees exhibit equally severe leaf symptoms when growing on either mazzard or mahaleb stock.

## SUMMARY

Mottle leaf, a virus disease of cherries that has been transmitted artificially only by grafting or by some adaptation of the grafting process, has been observed affecting cherry trees growing in Washington, Oregon, Idaho, California, and British Columbia. It is probable that the disease has been present in the Pacific Northwest for more than 20 years, although it was not proved to be bud- or graft-transmissible until 1935.

The leaves exhibit the most conspicuous symptoms of the disease, although the growth and fruit of the tree are also affected in instances where leaf symptoms are severe.

Considerable variation was found in the leaf symptoms of the different cherry varieties. Under field conditions only four varieties have been found with mottle leaf symptoms. Bing and Napoleon are the two most important commercial varieties that display severe leaf symptoms. Of the 19 cherry varieties inoculated none was immune to mottle leaf, although many were quite tolerant and showed very slight leaf symptoms.

Attempts to transmit the disease to other stone fruits have been mostly unsuccessful because the infective cherry buds did not form a growth union with them. However, under greenhouse conditions where growth union was established between cherry and peach, a preliminary experiment demonstrated that the virus was transmitted through tissues of the peach.

Mottle leaf was transmitted by budding from cherry to cherry, producing definite symptoms in 14 days on trees grown in the greenhouse and in 37 days under field conditions.

Tests demonstrated that mottle leaf could be transmitted by any method where growth union was established between diseased and healthy tissues. No mechanical method of transmission was found to be successful. The manner in which mottle leaf spreads in the field is unknown.

The black cherry aphid (*Myzus cerasi*) was tested as a possible vector of mottle leaf, and no transmission was obtained in these experiments.

Hot-water treatment of bud sticks at 46° C. for 60 minutes and at 49° for 10 minutes failed to inactivate the infective principle under the conditions of the experiment.

Cherry disorders causing leaf malformations that are sometimes confused with mottle leaf are briefly described.

There is no record of a tree having recovered from the disorder once the leaves definitely exhibit mottle leaf symptoms. It is suggested that cherry trees affected by mottle leaf be removed from the orchard to prevent the disease from spreading.

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## THE BORON DEFICIENCY DISEASE IN CABBAGE <sup>1</sup>

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### INTRODUCTION

As early as 1900, Copeland and Kahlenberg (9)<sup>2</sup> pointed out that the root development of lupine (*Lupinus albus* L.) seedlings seemed to be stimulated when a trace of boron was added to the water in which they were growing. Three years later Nakamura (20) reported stimulation of growth of peas (*Pisum sativum* L.) when borax was added to the soil at the rate of 1 mg. per kilogram. Agulhon (1) pointed out in 1910 that boron was a useful element to many plants, including radish (*Raphanus sativus* L.) and turnip (*Brassica rapa* L.). At about the same time in the Philippine Islands, Roxas (23), at the suggestion of Copeland, studied the effect of applying borax to potted soil in which rice (*Oryza sativa* L.) was planted. Although toxic effects resulted at some concentrations, it was observed that one application of M/100,000 boron stimulated leaf growth. In 1914 Brenchley (5) pointed out that in liquid-nutrient cultures the growth of peas was stimulated by certain very low concentrations of boron as boric acid, and in 1916 Moore (3) reported similar results with radish in both liquid- and sand-nutrient cultures.

Mazé (17, 18) in 1919 offered the first proof that traces of boron were necessary for the growth of corn (*Zea mays* L.). In 1923 Warington (26), working in Brenchley's laboratory, concluded that certain species of legumes require boron for normal development and that the absence or deficiency of this element in the nutrient solution brought about definite pathological symptoms. She did not prove it to be absolutely essential for barley or rye. At the present time it is rather generally accepted that boron is an essential minor element for most, if not all, higher plants and, furthermore, that certain species require relatively more than others for their normal development.

In 1930 Mes (19) pointed out that the symptoms of boron deficiency produced on tobacco in nutrient solution were similar to the top rot disease in Sumatra, and in the following year Brandenburg (4) offered proof that the long-recognized heart rot and dry rot of sugar beet (*Beta vulgaris* L.) is a boron-deficiency disease. After these two discoveries, numerous diseases of many crops were rapidly shown to be due to boron deficiency. In this list are various diseases of crucifers. MacLeod and Howatt (16), working in eastern Canada and reporting in 1935, were the first to show that brown heart of rutabaga (*Brassica campestris* var. *napobrassica* DC.) and turnip

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<sup>2</sup> Italic numbers in parentheses refer to Literature Cited, p. 586.

was reduced and often prevented entirely in the field by the application of borax to the soil. This result was confirmed during the same year in Wales (27), in Finland (14), and in Scotland (22). It was also in 1935 that the internal and external browning of the curd of cauliflower (*Brassica oleracea* var. *botrytis* L.) in the Catskill Mountain district of New York State was shown to be a boron-deficiency disease by Dearborn et al. (10, 11). This was confirmed by Hartman (13) on Long Island in 1936, by Chandler et al. (7) in Maine in 1937, and by Ferguson (12) in Canada in 1938. Hartman (13) also pointed out that cabbage (*B. oleracea* var. *capitata* L.) growing adjacent to severely affected cauliflower produced large marketable heads. Chandler et al. (6, 8) reported further that water-soaked brown areas developed in the fleshy stems of boron-deficient kohlrabi (*B. oleracea* var. *gongyloides* L.) and kale (*B. oleracea* var. *viridis* L.), while in sprouting broccoli (*B. oleracea* var. *botrytis* L.) abscission of buds occurred. Symptoms of boron-deficiency were observed in the field, however, only in cauliflower and turnip. When acute shortage of boron prevailed, as provided in controlled nutrient culture, the formation of storage organs of the cole group was suppressed or prevented.

The purpose of the present paper is to report experiments, conducted in both greenhouse and field, upon the internal break-down of cabbage and the closely related plants, kale, collard (*B. oleracea* var. *viridis* L.), and sprouting broccoli when boron deficiency in the soil occurs. Interest in this problem arose in connection with the internal break-down of cabbage heads affected with a mosaic disease (15).

#### METHODS AND MATERIALS

Plants were grown in the greenhouse at Madison, Wis., in sand cultures provided with a constant supply of nutrient by a modification of the method described by Allison and Shive (2). Clean 8-inch clay pots were varnished, provided with adequate drains, and filled with washed white quartz sand. The flow of nutrient was adjusted to deliver about 1 liter per day to each pot. Shive's Best three-salt solution<sup>3</sup> (25), to which traces of copper as sulfate ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ), iron as sulfate ( $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ), zinc as sulfate ( $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ), and manganese as chloride ( $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ ) had been added, was diluted 10 times with distilled water to provide a basic nutrient solution with a total salt concentration of 0.738 gm. per liter. This is referred to as the boron-free nutrient solution. Care was taken to avoid contamination with boron by using boron-free glassware. For the complete nutrient solution boric acid was added in amounts sufficient to provide 0.75 p. p. m. of boron. Seeds of cabbage (five varieties), turnip (two varieties), cauliflower, rutabaga, radish, and kohlrabi were sown directly in the pots. When plants were transferred from complete to boron-free nutrient and vice versa, first all were removed; some were transplanted back into the original pots, others were exchanged and transplanted. In this way both control and exchanged plants were comparable insofar as handling was concerned.

Field studies were carried out near Winneconne, Winnebago County, Wis., on Poygan silty clay loam with a soil reaction of about pH 7.5. The field had had an application of barnyard manure and 2 tons of

<sup>3</sup>The formula for this solution is: Monopotassium phosphate ( $\text{KH}_2\text{PO}_4$ ), 0.018 M; calcium nitrate ( $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ ), 0.0082 M; and magnesium sulfate ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ), 0.0150 M.

ground limestone per acre in the spring of 1936. In 1937 a crop of sugar beets grown on it had shown severe heart rot. The field experiments reported herein were conducted on this area in 1938, 1939, and 1940.

Plots 30 by 30 feet, with 5-foot borders, were laid out in 4 blocks of 9 treatments each, and the arrangement of the treatments was randomized within each block. Borax and salts of the other minor elements used were applied broadcast with the fertilizer by means of a lawn fertilizer drill, and harrowed thoroughly into the soil 5 weeks before the crops were planted.

## EXPERIMENTAL RESULTS

### SYMPTOMS ON SAND-NUTRIENT-CULTURE PLANTS

The expression of boron-deficiency symptoms on cabbage varies with the age of the plant and with the extent to which it is deprived of this element. When seedlings are grown in sand-nutrient culture from which boron is excluded, the disease develops rapidly and growth is sharply repressed. In soil in the greenhouse or in the field, boron starvation is neither so rapid nor so complete.

Symptoms of boron deficiency were produced experimentally on sand-nutrient-culture plants in the greenhouse. They will be described first. The field symptoms will then be discussed in comparison with those on cauliflower, kale, collard, and sprouting broccoli.

In sand-nutrient cultures, cabbage seedlings emerged 5 to 7 days after the seed was sown. When a boron-free nutrient solution was supplied the first effects were apparent in 7 to 10 days after emergence, usually most conspicuously on the first leaf, for the cotyledons as a rule expanded quite normally. The leaf blade became linear or circular in shape (fig. 1, *A* and *B*). Lateral venation was reduced and sometimes was almost entirely absent, while the leaf lamina may have split away from the midrib. The affected leaf was usually abnormally dark green and was somewhat thicker than the normal leaf. Early death of the growing point commonly occurred (fig. 1, *A* and *B*), and as growth of buds at the cotyledonary and other leaf nodes followed, the stunting of the plant was even more pronounced (fig. 2, *A*).

When plants started in a boron-free nutrient solution were transferred to a complete solution, rather prompt recovery occurred. New growth frequently took place from an axillary bud, as shown in figure 2, *C*. If, however, the growing point had not been killed new leaves formed normally. When seedling plants growing in a complete solution were transferred to a boron-free nutrient solution, the effects of the deficiency were apparent in 2 to 7 days. The leaves which had reached their full size were not affected, but the young unfolding ones at once began to assume the distorted morphology found in plants that start off in the deficient solution (fig. 2, *B*). Seedlings of cauliflower, kohlrabi, rutabaga, turnip, and icicle radish responded very similarly to cabbage when they were grown in boron-free nutrient solution.

The symptoms described above are those resulting when conditions for complete boron starvation of young seedlings are provided. When plants of cabbage are allowed to grow to greater size in a complete nutrient solution and the boron supply is then greatly reduced without

being completely excluded, leaf malformation is again the chief sign of deficiency but internal break-down of the pith may also occur. Variation between individuals in the expression of abnormal growth is more noticeable in the somewhat older plants than in young seedlings. In figure 3 the leaves from plants grown in a boron-deficient nutrient solution are compared with those on plants of the same age grown continuously in the complete solution. The plants were started from seed in complete nutrient solution. After 17 days the boron supply was reduced to a very slight trace for some plants. Signs of boron deficiency appeared in 10 days, and although the growth rate was not materially reduced, various manifestations con-

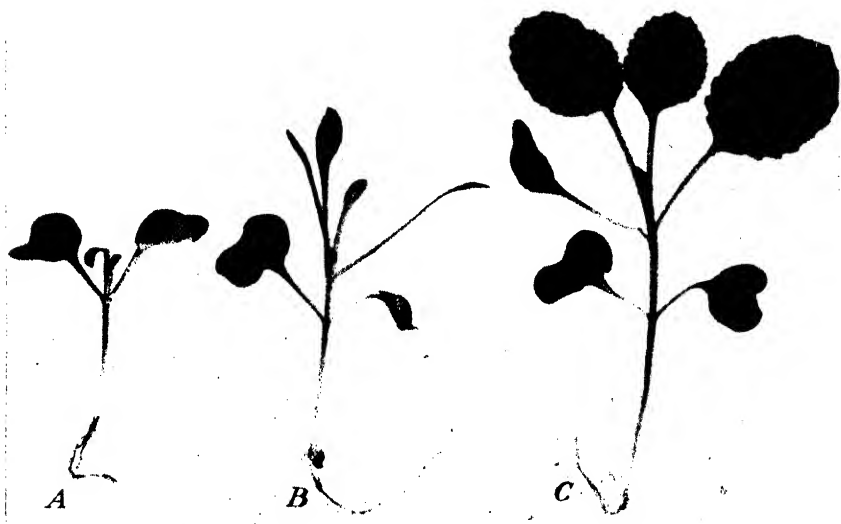


FIGURE 1.—Cabbage seedlings (Jersey Queen variety) grown from seed in sand-nutrient culture for 31 days: *A* and *B*, grown in boron-free nutrient solution; *C*, grown in complete nutrient solution. Note that the cotyledons are normal in all three, but in the boron-deficient plants the growing points are killed and the leaves are dwarfed and distorted.

tinued to develop during several weeks. One of the signs of boron deficiency is a darker green color of one or more leaves; later the plant may produce more normally colored leaves. Puckering of the leaf blade in various ways, but more commonly near the midrib, may follow (fig. 3, *B*, *C*). This distortion is the result of one or both of two causes: (1) The retardation of growth of the tissue near the margin of the leaf and (2) stimulation of excess growth in the region of the midrib not unlike that discussed by Van Schreven (24). If the lack of boron affects the leaf earlier in its development, linear formation accompanied by curling from the suppression of marginal growth follows (*C*, *F*); or even greater stunting and malformation may result in lateral distortion or suppressed lateral venation with segments of the leaf entirely absent (*D*, *E*). Occasionally cross hatching of the upper surface of the midrib occurs (*G*), resembling that commonly associated with boron deficiency in sugar beet (4). An interveinal marginal yellowing sometimes appears on the older leaves of boron-

deficient plants (*H, I*) after symptoms of malformation have appeared. Yellowing may appear later on the younger leaves.

#### SYMPTOMS ON FIELD-GROWN PLANTS

It should be emphasized that it was commonly the case that no external symptoms of boron deficiency in cabbage were seen in the field even when the crop was grown on soil where sugar beet, table beet, and cauliflower showed external signs. This is in accord with the observations of Hartman (13), cited above. The field in which

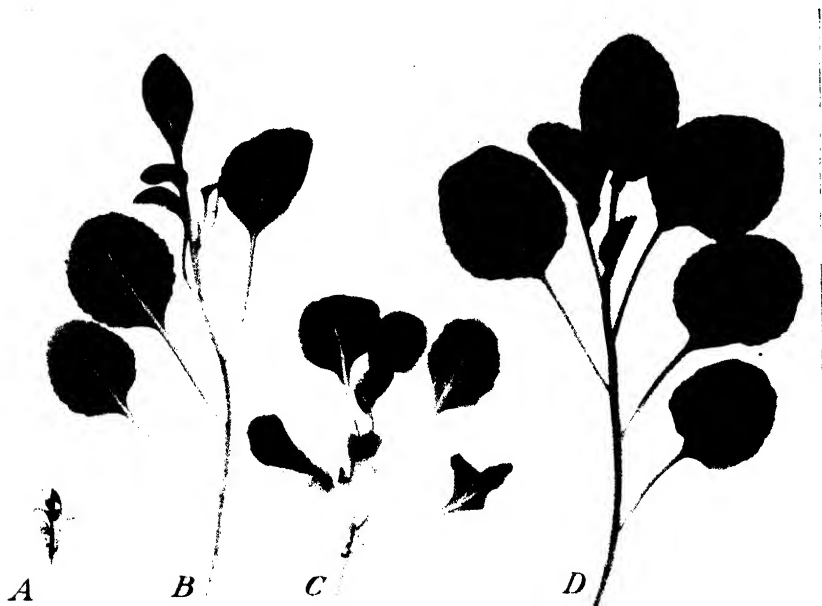


FIGURE 2. Cabbage seedlings (Jersey Queen variety) grown in sand-nutrient culture: *A*, Grown for 73 days from seed in boron-free nutrient solution; *B*, grown in complete nutrient solution for 33 days, then transplanted to boron-free culture and continued for 40 days (note distortion of the youngest leaves); *C*, grown for 33 days in boron-free nutrient solution, then transplanted to complete solution and continued for 40 days (note that the growing point was killed and that the new growth proceeded normally from a leaf node); *D*, grown in complete solution for 73 days.

the following observations were made was one in which heart rot of sugar beet was severe in 1939. The appearance of cauliflower in midseason in that year is shown in figure 4. No external symptoms were found in cabbage in 1939, and the size, solidity, and weight of cabbage heads were not noticeably different in plants grown on this soil from those grown on the same soil where 40 pounds of borax per acre had been applied, a treatment that corrected the disease in sugar beet and cauliflower. In 1940 cabbage transplanted to these plots in June showed in mid-August moderate symptoms on the outer leaves with occasional stunting in growth. The symptoms included mottling along the margins of the oldest leaves, cross hatching on the upper surface of the petiole and midrib, abnormal thickening



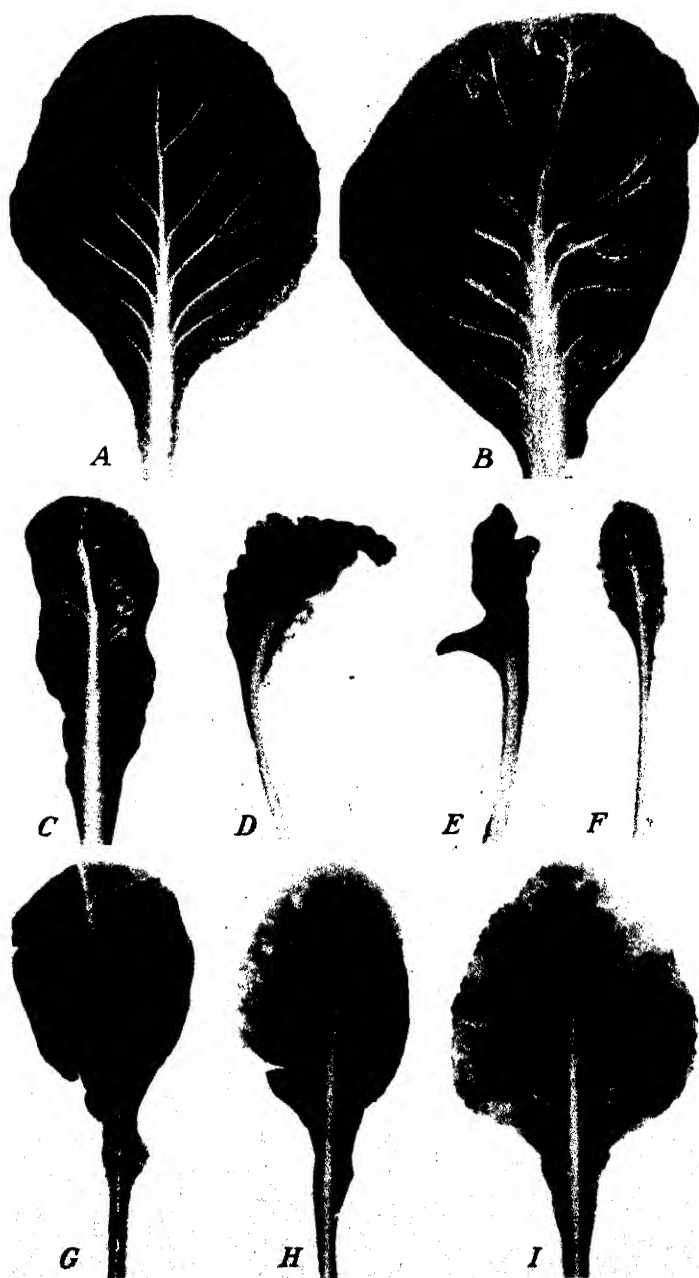


FIGURE 3.—A, Leaf from plant grown in complete solution; B to I, leaves from plants grown with a reduced boron supply. Photographed when plants were 120 to 130 days old. (See further explanation in text.)



FIGURE 4.—*A*, Cauliflower grown on boron-deficient soil; *B*, comparable plant grown on an adjoining plot to which 40 pounds of borax per acre was applied before planting. Note reduced size of diseased plant (*A*), downward marginal curl of oldest leaves resulting from suppressed marginal growth, and reduced size and distortion of the youngest leaves.

of the leaf lamina, unilateral leaf development, and curling of the leaf margin. Younger leaves usually developed without symptoms.

When mature cabbage plants grown on boron-deficient soil were examined by making a vertical cut through the head so as to bisect the main stem longitudinally, the pathological symptoms were found in the pith of the stem. They were not unlike those described by Dearborn et al. (10, 11) in cauliflower stems. Water-soaked spots developed first, and the tissue therein gradually turned brown while the lesions enlarged. Necrotic spots thus formed, usually midway between the top of the core and the base of the head (fig. 5), but often extending well into the lower stem. Sometimes the break-down increased to a point where a cavity formed (fig. 6). This symptom, however, is relatively rare in cabbage under the same conditions in which it is common in cauliflower. No necrosis that can be attributed to boron deficiency has been noted in the leafy portion of cabbage heads except in the midrib adjacent to the main stem. The break-down in the core is of definite economic importance, particularly in the manufacture of sauerkraut, where the shredded pith is used as well as the leafy portion and where the discolored, corky, necrotic tissue detracts from the quality of the final product. Similar internal necrotic areas occurred in the field on kale, collard, and sprouting broccoli. The symptoms described are in general accord with those reported recently from Maine by Chandler (6).

#### RELATIVE SUSCEPTIBILITY OF SUBSPECIES OF *BRASSICA OLERACEA*

Seed of several members of the *cole* group was sown in the boron-deficient soil at Winneconne on May 25, 1938. The 30-foot single-row plots of each lot were placed in random arrangement in each of three blocks. The plants were thinned to 2-foot intervals on June 20, leaving 15 plants per row plot. On August 31 all cauliflower plants were sectioned and examined; the other subspecies were so handled on September 24. The varieties used and the results secured are given in table 1.

TABLE 1.—*Development of internal break-down in subspecies of Brassica oleracea grown on boron-deficient soil at Winneconne, Wis., 1938*

[15 plants used in each test]

Subspecies and variety	Plants showing internal break-down in—			
	Replicate 1	Replicate 2	Replicate 3	Average
	Percent	Percent	Percent	Percent
Cauliflower (Snowball).....	93.3	93.3	73.3	86.6
Cabbage (Marion Market).....	6.7	13.3	20.0	13.3
Broccoli (Green Sprouting).....	0	33.3	0	11.1
Collard (Cabbage).....	0	13.3	33.3	15.5
Kale (Thousand-headed).....	46.7	20.0	20.0	28.9

While the differences between cabbage, broccoli, collard, and kale were not significant, it is very evident that even though they were allowed to stand approximately a month longer than the cauliflower, as a group they showed much less of the disease than the cauliflower. Moreover, the full extent of the difference is not expressed in percentage of plants affected, for the degree of break-down was also correspondingly greater in cauliflower than in the other forms.

## RELATIVE SUSCEPTIBILITY OF CABBAGE VARIETIES

Several varieties of cabbage, representing a range from the early-maturing type, Golden Acre, to the late-storage form, Wisconsin

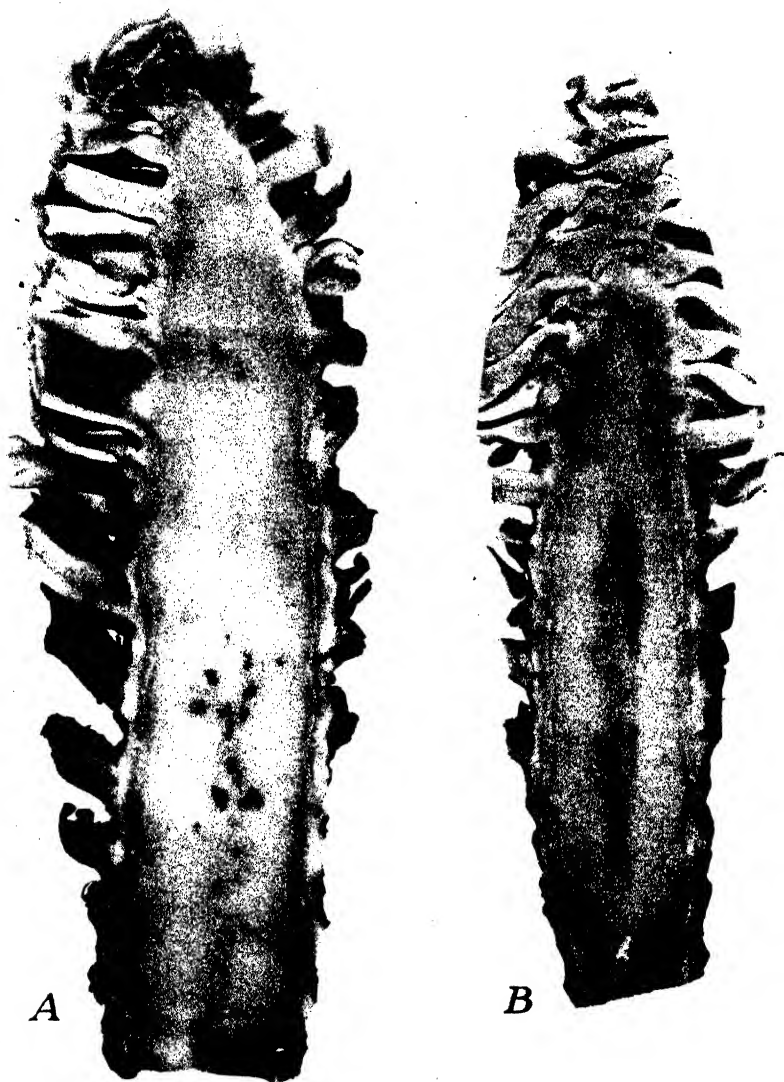


FIGURE 5.—Internal necrosis of stem of cabbage grown on boron-deficient soil: A, Scattered necrotic spots; B, a more extensive area with concentrated necrotic tissue.

Hollander, were transplanted to the Winneconne field in the latter part of June of 1939 and 1940. The seedlings had been grown in the seedbed at Madison on soil not known to be boron-deficient.

For each variety a single-row plot of 30 plants in 1939 and of 15 plants in 1940 was placed at random in each of 3 blocks. All plants grew without external symptoms in 1939, but in 1940 symptoms



FIGURE 6.—Extreme stage of internal break-down of cabbage grown on boron-deficient soil.

already described occurred on the outer leaves of some plants. As the heads became mature they were sliced and the extent of internal break-down was recorded (table 2).

TABLE 2.—*Occurrence of internal break-down in several varieties of cabbage and in cauliflower grown on boron-deficient soil at Winneconne, Wis., 1939 and 1940*

[Average of 3 replicates in each year]

Subspecies and variety	1939		1940		
	Date of harvest	Internal break-down	Date of harvest	Internal break-down	Arc sin $\sqrt{x}$ radians
		Percent		Percent	
Cauliflower, Snowball			Sept. 19	98	1.4162
Cabbage:					
Jersey Queen			Oct. 7	6	.2505
Golden Acre	Sept. 14	67	do	60	.8870
Resistant Detroit	do	70	do	62	.9033
Racine Market	do		Oct. 17	43	.7126
Marion Market	Oct. 3	45	do	42	.7034
All Head Select	Oct. 26	26	do	15	.3993
Wisconsin All Seasons	do	36	do	10	.3158
Wisconsin Ballhead	do	33	do	33	.6134
Wisconsin Hollander	do	33	do	32	.6030
Resistant Red Hollander			do	58	.8705
Difference required for significance between varieties (19:1)		25			.3804

Of the white varieties, Golden Acre, Resistant Detroit, and Marion Market contained in both seasons the largest percentages of plants with internal break-down. In 1940 Racine Market was in this group. All four of these varieties are genetically related, since they were each derived by selection from Copenhagen Market. All Head Select, Wisconsin All Seasons, Wisconsin Ballhead, and Wisconsin Hollander were less seriously affected than the Copenhagen group.

The data in 1939 suggested that, since the earliest maturing varieties were the most seriously affected, a correlation between earliness and susceptibility might exist under these growing conditions. However, this does not hold in the 1940 data. Jersey Queen although of the same maturity as Golden Acre had significantly less disease than the latter, while Resistant Red Hollander of the same season as Wisconsin Hollander was about as seriously affected as Golden Acre. Cauliflower was distinctly more susceptible than all cabbage varieties tested.

#### CONTROL THROUGH SOIL TREATMENT

The proof that the internal break-down of cabbage was the result of boron deficiency in the soil was amplified by soil-treatment experiments conducted at Winneconne in 1938 and 1939. As already indicated, four replicate blocks were laid out in which treatment plots were placed at random. In 1938, because of other experimental work under way, it was not possible to include cabbage in all of these. However, Wisconsin All Seasons cabbage was planted in each plot of two of the blocks and Snowball cauliflower in each plot of the other two blocks. The various treatments and the percentage of plants showing internal break-down in each plot are given in table 3. As a rule, the disease was less serious in cabbage than in cauliflower. The percentage of affected plants was reduced very greatly when 20 pounds of borax per acre was applied. In the case of cauliflower, the disease was eliminated in the 40-pound and 60-pound plots, but a small percentage of diseased plants of cabbage remained in these treatments. Manganese sulfate, applied separately, seemed to have

no effect on the correction of internal break-down, nor did it have any consistent effect when used to supplement borax. The other minor elements tested likewise had no perceptible effect upon the reduction of the disease.

TABLE 3.—*Effect of borax and salts of certain other minor elements on the occurrence of internal break-down of cabbage and cauliflower at Winneconne, Wis., 1938*  
[15 plants used in each test]

Soil treatment			Internal break-down in—					
Fertilizer (3-12-12) per acre	Borax per acre	Manganese sulfate per acre	Cabbage			Cauliflower		
			Block 1	Block 2	Average	Block 1	Block 2	Average
Pounds	Pounds	Pounds	Percent	Percent	Percent	Percent	Percent	Percent
0	0	0	(1)	20.0	20.0	86.7	40.0	63.3
450	0	0	(1)	26.7	26.7	66.7	53.3	60.0
450	0	100	73.3	53.3	63.4	13.3	100.0	56.7
450	20	0	0	20.0	10.0	6.7	0	3.4
450	20	50	0	0	0	0	6.7	3.4
450	20	50	0	6.7	3.4	40.0	6.7	23.4
450	40	0	0	6.7	3.4	0	0	0
450	40	50	6.7	(1)	6.7	0	0	0
450	60	0	6.7	0	3.4	0	0	0

<sup>1</sup> No notes were taken on account of water damage.

<sup>2</sup> Also 50 pounds each per acre of copper sulfate, iron sulfate, and zinc sulfate, and 100 pounds per acre of sodium chloride.

In 1939 the same blocks were used without further treatment except for the application of 300 pounds per acre of 3-12-12 fertilizer to each plot but the one in each block to which no fertilizer had been applied in 1938. Two varieties of cabbage, Marion Market and Wisconsin Ballhead, were planted throughout. The results at the end of the season are given in table 4. The percentages of internal break-down were very similar to those recorded in 1938, confirming the indication that boron deficiency is the cause of the break-down. It is of interest to note that in this soil the boron still available from the treatment of the previous year was sufficient to correct the disease as effectively as it had in that year.

TABLE 4.—*Effect of borax and salts of certain other minor elements applied at Winneconne, Wis., in the spring of 1938, on the occurrence of internal break-down in the cabbage crop of 1939*

Soil treatment				Internal break-down in—		
Fertilizer (3-12-12) per acre—		Borax per acre, 1938	Manganese sulfate per acre, 1938	Marion Market	Wisconsin Ballhead	Average
1938	1939					
Pounds	Pounds	Pounds	Pounds	Percent	Percent	Percent
0	0	0	0	<sup>1</sup> 13.3	<sup>1</sup> 11.7	12.5
450	300	0	0	25.0	16.7	20.9
450	300	0	100	26.3	21.7	24.0
450	300	20	0	0	1.7	.9
450	300	20	50	3.3	3.3	3.3
450	300	20	<sup>2</sup> 50	1.7	1.7	1.7
450	300	40	0	3.3	0	1.7
450	300	40	50	0	0	0
450	300	60	0	1.7	0	.9

<sup>1</sup> Average of 4 replicates of 15 plants each.

<sup>2</sup> Also 50 pounds each per acre of copper sulfate, iron sulfate, and zinc sulfate, and 100 pounds per acre of sodium chloride.

## DISCUSSION

Internal break-down of the tissue of cabbage heads is a factor that may detract greatly from the quality of the final product. Suboxidation in storage and transit has been shown by Nelson (21) to be a causal factor. More recently Larson and Walker (15) have associated symptoms very similar to those described by Nelson (21) with a mosaic disease. The present study adds another causal factor that may be responsible for an internal necrosis in the cabbage head.

It is of importance to note that the internal break-down in cabbage due to boron deficiency appears to occur largely in the pith of the main stem or core. In this regard it is distinct in location from the other two diseases mentioned. Furthermore, cabbage, as judged by the varieties tested, is much less susceptible than cauliflower. On the other hand, the usual lack of distinguishable external symptoms in the field places the disease among those that are not ordinarily detected until the product has reached the final culinary or processing stages.

The fact that varieties seem to differ in their response to boron deficiency in the soil would seem to justify further inquiry into this phase of the disease. It is also equally evident that such a genetic tendency should be kept under consideration in any program of improvement of this crop plant through breeding.

The disease appears to be readily corrected by the addition of borax to the soil. However, it is significant that, in the limited number of experiments reported, the complete elimination of the internal necrosis in cabbage was not accomplished even at 60-pound-per-acre applications, while the more susceptible cauliflower was completely healthy after a 40-pound-per-acre treatment. It remains to be determined whether the small amount of necrosis that persisted was due to other factors, or whether the cauliflower plant has properties that result in greater efficiency in the use of the boron salt applied to the soil.

## SUMMARY

The symptoms of boron deficiency in young cabbage plants growing in sand-nutrient cultures in the greenhouse are described.

On boron-deficient soil the field-grown crop often showed no external signs of disease. Internal break-down of the pith was the chief symptom in the tissue.

Cauliflower was found to be much more subject to internal break-down on boron-deficient soil than cabbage, kale, collard, or sprouting broccoli.

Of several varieties of cabbage tested on boron-deficient soil the early-maturing ones, Golden Acre and Resistant Detroit, showed the greatest amount of internal break-down.

Applications of 20 pounds or more of borax per acre practically though not entirely eliminated the disease.

These applications of borax were equally effective when the second successive crop of cabbage was grown, without further treatment of the soil with borax.

Salts of manganese, copper, iron, zinc, and sodium applied to the soil had no effect upon the occurrence of internal break-down.



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# A BLUE STAIN FUNGUS, *CERATOSTOMELLA MONTIUM* N. SP., AND SOME YEASTS ASSOCIATED WITH TWO SPECIES OF *DENDROCTONUS*<sup>1</sup>

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## INTRODUCTION

Blue stain is a concomitant of the infestation of trees by certain bark beetles. Stains associated with some species of beetles have been found in previous investigations to be caused by species of *Ceratostomella*, which are carried into galleries mined by the attacking insects and grow in the cells of the sapwood (3, 11, 13, 15, 16).<sup>3</sup> It is believed that these fungi may be an important factor in causing the death of the trees. An investigation of the possible association of fungi with *Dendroctonus monticolae* Hopk. and *D. ponderosae* Hopk. resulted in finding a new species of *Ceratostomella*, herein described, which causes the blue stain. A study was made also of some yeasts that are apparently associated with the beetles.

*Dendroctonus monticolae* is one of the destructive insects of the western forests. It is found killing pines in the Sierras of California from the south to the north, and in the forests of Oregon, Washington, Idaho, Montana, and western Canada. The beetle attacks living trees and yearly with its associated fungus kills many acres of lodgepole pine (*Pinus contorta latifolia* Engelm.), possibly accounting for the relatively short life of these trees (4). It also kills sugar pine (*P. lambertiana* Dougl.), western white pine (*P. monticola* Dougl.), and ponderosa pine (*P. ponderosa* Dougl.).

*Dendroctonus ponderosae* also is regarded as one of the most aggressive and destructive of bark beetles. It is found in the mountain forests of western South Dakota, southeastern Montana, Wyoming, Colorado, Utah, and northern Arizona and New Mexico. Its favorite host is ponderosa pine, but it infests other pines, such as lodgepole and limber pine (*Pinus flexilis* James), when they occur with ponderosa (1). It was the cause of two historic epidemics recorded by the early settlers of the Rocky Mountain region, when acres of trees were killed in the Black Hills and Kaibab National Forests. Investigations by entomologists show that there have been a series of bark beetle epidemics extending through the years. The examination of a 400-year-old pine showed by the pitch pockets in its wood that it had survived seven series of unsuccessful beetle attacks. Hopkins (9) wrote:

Very conclusive evidence has also been found that some of the great denuded areas in the Rocky Mountain region supposed to have been caused by forest fires were primarily caused by one or more species of *Dendroctonus*.

<sup>1</sup> Received for publication December 31, 1940.

<sup>2</sup> The investigations on which this paper is based were conducted in cooperation with the Division of Forest Insect Investigations, Bureau of Entomology and Plant Quarantine, and the Forest Products Laboratory, Forest Service, U. S. Department of Agriculture.

<sup>3</sup> Italic numbers in parentheses refer to Literature Cited, p. 600.

## MATERIALS AND METHODS

The specimens studied were collected by entomologists, who selected specimens of *Dendroctonus monticolae* within the region in the northern Rocky Mountains where this bark beetle is distributed, and of *D. ponderosae* within its Rocky Mountain region. They also furnished specimens collected in areas outside of their recognized territories and in territories where some of the beetles are atypical.<sup>4</sup>

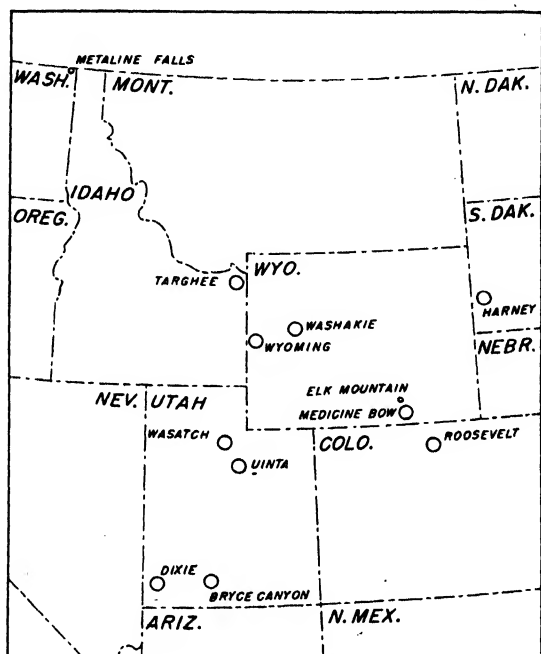


FIGURE 1.—Map of regions where bark beetles and blue-stained wood were collected.

Figure 1 shows the location of forests where collections were made of bark beetles and blue-stained wood used in the investigations here reported. As in previous studies (15, 16), cultures were started by transferring to malt agar<sup>5</sup> insects, eggs, larvae, gallery walls, frass, or fragments of blue-stained bark, and wood. As soon as ascospores were produced they were planted, by the dilution method, on malt-agar plates from which single colonies were transferred to malt-agar slants for use in this study. The final selection of the isolate from its group depended on its vigor of growth and fructification. The isolates of *Ceratostomella* used in describing the fungus and for taking measurements were grown on malt agar and kept where the temperatures ranged from 12° to 17° C.

Table 1 gives the source and history of the isolates and dimensions of the perithecia when grown on malt agar.

<sup>4</sup> Entomologists record as atypical those specimens of a bark beetle infestation which do not show all the usual characteristics of a species.

<sup>5</sup> Formula for malt agar: Malt extract (Trommer's plain), 25 gm.; agar, 15 gm.; and water, 1,000 cc. The acidity is pH 5.5 to 5.6.

TABLE 1.—Dimensions of perithecia of *Ceratostomella montium* isolated from *Dendroctonus monticolae*, *D. ponderosae*, or wood infested with them, and grown on malt agar

Source of culture	Fruits measured	Height of base		Width of base		Length of neck	
		Usual range <sup>1</sup>	Mean	Usual range	Mean	Usual range	Mean
<i>Dendroctonus monticolae</i> , in region of usual distribution, on—	Number						
<i>Pinus monticola</i> , Metaline Falls, Wash.	71	249-363	277	240-308	268	1, 158-1, 762	1, 445
<i>P. contorta latifolia</i> , Targhee National Forest, Idaho	573	240-319	279	226-311	267	1, 167-1, 817	1, 487
<i>P. flexilis</i> , Washakie National Forest, Wyo.	73	260-329	296	232-319	278	1, 217-2, 211	1, 681
<i>P. contorta latifolia</i> , Wyoming National Forest, Wyo.	4	246-260	260	250-261	260	1, 516-1, 600	1, 532
Total, range, or mean (14 cultures)	721	243-320	280	228-311	268	1, 170-1, 853	1, 503
<i>Dendroctonus monticolae</i> , south of its recognized territory, on—							
<i>Pinus contorta latifolia</i> , Provo River area, Wasatch National Forest, Utah	359	275-356	314	258-340	299	1, 190-2, 001	1, 581
<i>P. contorta latifolia</i> , Tabbie Mountain, Uinta National Forest, Utah	110	274-348	309	257-336	297	1, 228-2, 130	1, 657
Total, range, or mean (4 cultures)	469	274-353	312	258-339	298	1, 190-2, 020	1, 599
<i>Dendroctonus ponderosae</i> , in region of usual distribution, on—							
<i>Pinus ponderosa</i> , Divisadero National Forest, Utah	187	246-328	286	231-317	275	1, 004-1, 451	1, 251
<i>P. ponderosa</i> , Bryce Canyon National Park, Utah	191	245-336	288	229-321	277	1, 010-1, 737	1, 410
<i>P. ponderosa</i> , Roosevelt National Forest, Colo.	75	239-300	272	231-308	267	923-1, 215	1, 044
<i>P. ponderosa</i> , Harney National Forest, S. Dak.	90	253-335	289	241-318	280	992-1, 490	1, 227
Total, range, or mean (11 cultures)	543	245-330	285	231-318	275	979-1, 771	1, 277
<i>Dendroctonus ponderosae</i> , territory where some of the beetles are atypical, <sup>2</sup> on—							
<i>Pinus flexilis</i> , Elk Mountain, Carbon County, Wyo.	264	256-333	293	240-325	281	1, 168-1, 857	1, 521
<i>P. contorta latifolia</i> , Elk Mountain, Carbon County, Wyo.	379	243-322	282	230-309	269	1, 202-2, 048	1, 621
<i>P. ponderosa</i> , Medicine Bow National Forest, Wyo.	289	262-324	293	252-311	281	1, 430-2, 204	1, 820
Total, range, or mean (22 cultures)	932	250-328	288	240-317	276	1, 241-2, 053	1, 654
Total, usual range, or mean for all strains	2, 665	250-330	287	228-320	278	1, 141-1, 902	1, 527

<sup>1</sup> Usual range discards the sixth of the measurements at each end of the extreme range, permitting an approximation of the frequency distribution.

<sup>2</sup> See footnote 4, p. 590.

### THE FUNGUS IN ITS NATURAL HABITAT

Both *Dendroctonus monticolae* and *D. ponderosae* bore through the outer bark of the living tree to the region of the cambium and there extend their galleries. The first galleries in the inner bark and cambium are long vertical tunnels mined by the adults; from these extend smaller horizontal galleries made by larvae. These have the effect of girdling the tree.

The fungus begins its growth in the galleries and penetrates the inner bark and wood. Perithecia develop on the walls of the galleries. The bases of these fruits are generally buried in the bark, wood, or packed frass, and their necks extend into the galleries (fig. 2, A). They develop in both the beetle and larval galleries, where active beetles and larvae are smeared with the spores. The fungus grows in toward the heartwood, staining the sapwood.

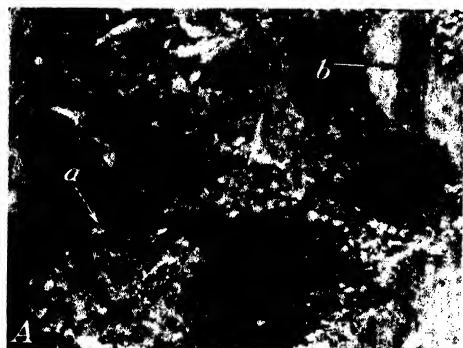


FIGURE 2.—A, Galleries of *Dendroctonus monticolae* in inner bark of *Pinus contorta latifolia*: a, Necks of perithecia extending into the galleries, with clumps of white ascospores on the tips of the necks; b, blue stain spreading from the galleries.  $\times 15$ . B, Tangential section of *P. contorta latifolia* sapwood infected with *Ceratostomella montium* associated with *D. ponderosae*. Blue stain hyphae in rays and tracheids.  $\times 400$ .

The color of the stain was not affected by the species of bark beetle or of the infested pine (*Pinus contorta latifolia*, *P. monticola*, *P. ponderosa*, or *P. flexilis*). Depending on the age of the infection and the moisture in the specimen, the colors ranged from Hathi gray (14) to storm gray, castor gray, dusky green gray, and blackish green gray. The color of the stain in the wood when received was usually castor gray. The rays become black.

As the fungus grows from the insect galleries it first invades the ray cells of the sapwood and later the tracheids, passing from cell to cell through the pits (fig. 2, B). Occasionally the hyphae penetrate the cell walls.

## THE FUNGUS IN CULTURE

### GROWTH

The young colonies are white. When the hyphae are about 6 days old their color begins to change to a warm sepia. Gradually the depressed mycelium turns black, while often light-gray aerial mycelium grows over part of the mycelial surface. On malt-agar slants the fungus often develops a thick white or cream-colored aerial mycelium at the top of the slant, which with age occasionally changes color to cartridge buff. As the culture ages, this aerial mycelium changes to wood brown in color or dries and becomes depressed. Some of this mycelium produces innumerable conidia on simple conidiophores; some of it is sterile. In the center of the slant the mycelium is olive brown,

then black. The necks of perithecia often extend through the blotches of dark-gray aerial mycelium. Mycelium has been found growing  $1\frac{1}{2}$  inches below the surface of the medium.

#### MYCELIUM

The hyaline hyphae in the young culture are septate, branch irregularly, and measure  $1.3\mu$  to  $4\mu$  in diameter. They form strands as they age, anastomose, and turn brown. Cell divisions become numerous, so that old hyphae sometimes consist of rows of short, slightly globular cells. The walls thicken, and sometimes a dark granular exudation appears. Old brown hyphae may be relatively thick, varying from  $4\mu$  to  $8\mu$  in diameter.

#### CONIDIA AND CONIDIOPHORES

The hyaline conidia, globular or ovoid, usually start developing 24 hours after the ascospore germinates. The globular conidia grow in

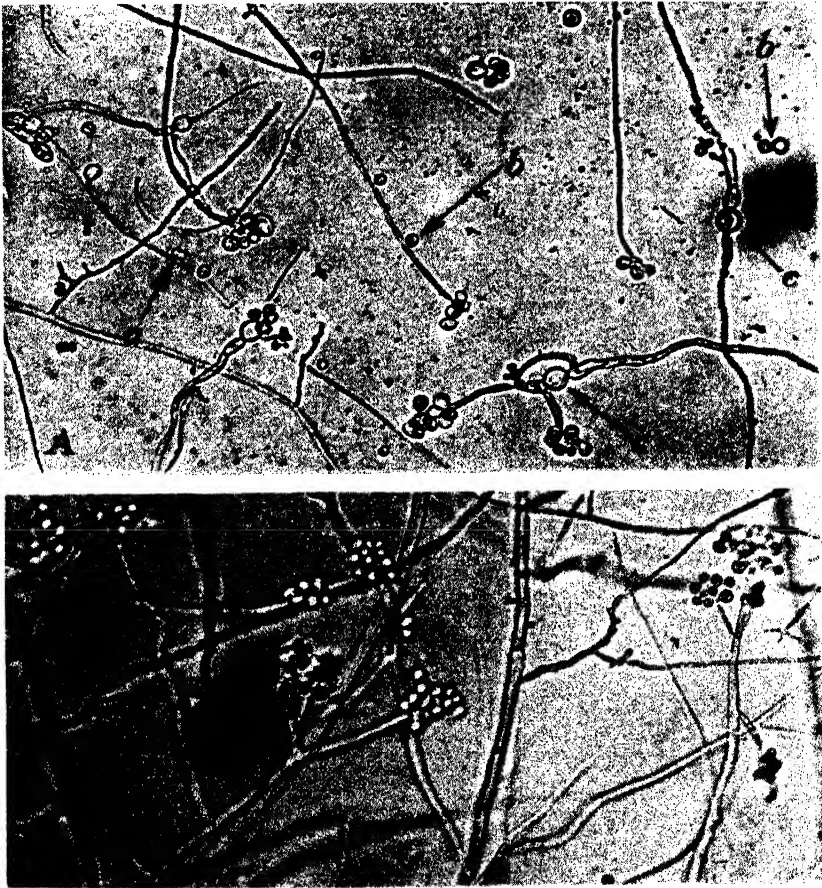


FIGURE 3.—Van Tieghem cell culture. A, Two days old, showing (a) ascospore, rectangular parallelepipedal with square ends; (b) globular ascospores; and (c) germinated ascospores with conidia developing in clusters on the hyphae.  $\times 360$ . B, Four days old, showing simple branching of conidiophores and clusters of conidia.  $\times 420$ .





FIGURE 4.—A, Perithecia on and in the body of a larva of *Dendroctonus monticolae*. The live larva was taken from a gallery mined under the bark of a lodgepole pine and was dropped into a test tube containing malt agar.  $\times 14$ . B, A section through the interior of the base of a young perithecium: a, Immature ascospores developing outside the ascus; b, somewhat older ascospores changing from globular to cylindrical; c, almost mature ascospore changing in shape to a rectangular parallelepiped with square ends.  $\times 1,750$ .

small clusters on the tips of short conidiophores or on the ends of hyphae and are  $4\mu$  to  $5\mu$  in diameter. Ovoid conidia,  $4\mu$  to  $5\mu$  wide by  $6.5\mu$  to  $8\mu$  long, form on the germ tubes, directly on the hyphae, or on short conidiophores (fig. 3, A and B).

When 1 month old, Van Tieghem cell cultures had produced increased numbers of conidia in the clusters and along the sides of the hyphae but the conidiophores remained simple. No culture that was started from a single conidium produced perithecia. About 30 such cultures were made from one isolate.

#### PERITHECIA

The perithecia of this fungus are the largest of any *Ceratostomella* so far found associated with bark beetles.

The largest and most abundantly sporulating perithecia developed on the bodies of adults that were dropped into test tubes containing malt agar, where they were used as inocula. Live larvae were also good inocula and substrata (fig. 4, A). When the cultures were 6 months old it was difficult to dissect the perithecia from the insects because of the toughness of the mycelium in the body. In 9-month-old cultures the beetle and larvae bodies were partly digested. Usually after the cultures were 1 year old the bodies of beetles and larvae had been digested, and it was fairly easy to remove the whole perithecium from the black mycelium.

The shape of the perithecium is typical of *Ceratostomella*. The black or dark-brown globular base is usually submerged partly or wholly in the substratum on which it is growing and is commonly bare of basal hyphae except those that attach it to the substratum (fig. 4, A). However, perithecia are found occa-

sionally in culture with basal hyphae growing into the air, looking like bristles. These are short, straight, septate, and brown, and taper at the tip.

The long slender neck is black or dark brown, usually lighter colored at the tip. Rarely it divides into two or three necks. Three rows of closely packed ascospores can pass through the neck at once. The ostiole is without cilia. The dimensions of the perithecia are given in table 1. In general, the bases of the perithecia in the strains associated with *Dendroctonus monticolae* and *D. ponderosae* in the regions of their usual distribution and beyond did not differ significantly in size. The necks were slightly shorter in those strains associated with *D. ponderosae*, but this difference is not regarded as significant because the lengths of necks of *Ceratostomella* perithecia are usually more variable than the sizes of the bases.

#### ASCI

The immature asci seen were clavate or ellipsoid and contained eight immature ascospores. The ascus wall disappears before the ascospores are mature (fig. 4, B). The ascospores develop to a large extent outside the ascus. Their formation appears to keep pace with the growth of the perithecial neck, as was the case with other insect-blue stain fungi studied previously (15, 16).

#### ASCOSPORES

The hyaline ascospores have the shape of rectangular parallelepipeds with square ends, and their angles are made prominent by flanges on the outer walls. They are covered with a thin coating of a mucilaginous substance. The ascospores resemble those of *Ceratostomella ips* Rumbold, another blue-staining fungus associated with insects (16, fig. 5).

Table 2 gives the dimensions of the ascospores of 20 isolates from different localities. The fungus cultures had been grown under similar laboratory conditions and were of about the same age when the perithecia were mounted for study. The length of the ascospores of all the isolates was practically the same; the width of the ascospores of the isolates from pines infested with *Dendroctonus ponderosae* in the region where this insect is usually distributed was about 4.6 percent smaller than those of the three other groups. Mathematical study showed that this difference in width is so small that it could be found between geographic strains of the same fungus even when associated with the same substratum and carrier. It is too small to use as a basis for distinguishing two species of fungi.

The ascospores did not germinate in sterile distilled water, but about 50 percent germinated within 24 hours in 3-percent malt solution or on malt agar. When ready to germinate the right square prism-shaped spore swells to more than twice its original size, becomes oval or round, and will produce 1 to 4 germ tubes (fig. 3, A, b and c).

In 2-day-old Van Tieghem cell cultures one finds ungerminated ascospores as well as some ascospores which have swollen and some which have germinated and produced hyphae and conidia. Figure 3, A, also illustrates the difference in size between germinated and ungerminated ascospores.

TABLE 2.—Dimensions of ascospores of *Ceratostomella montium* associated with *Dendroctonus monticolae* and *D. ponderosae*

Source of culture	Length of ascospore <sup>1</sup>		Width of ascospore	
	Usual range	Mean	Usual range	Mean
<i>Dendroctonus monticolae</i> :				
In region of usual distribution:	$\mu$	$\mu$	$\mu$	$\mu$
Metaline Falls, Wash.	4.4-5.1	4.6	2.4-3.0	2.8
Targhee National Forest, Idaho.	4.4-5.1	4.6	2.4-3.0	2.7
Do.	4.4-4.7	4.6	2.4-3.0	2.8
Do.	4.1-5.4	4.7	2.4-3.0	2.8
Washakie National Forest, Wyo.	4.4-4.7	4.5	2.7-3.0	2.8
Do.	4.4-5.1	4.7	2.4-3.0	2.6
Wyoming National Forest (Greys River), Wyo.	4.4-5.1	4.7	2.4-3.0	2.6
Total of 140 measurements, usual range, mean.	4.4-5.1	4.6	2.4-3.0	2.7
South of its recognized territory:				
Uinta National Forest, Utah.	4.4-5.1	4.8	2.4-3.0	2.7
Do.	4.1-5.1	4.6	2.4-3.0	2.8
Wasatch National Forest, Utah.	4.4-5.1	4.7	2.4-2.7	2.6
Do.	4.4-5.1	4.6	2.4-3.0	2.7
Total of 80 measurements, usual range, mean.	4.4-5.1	4.7	2.4-3.0	2.7
<i>Dendroctonus ponderosae</i> :				
In region of usual distribution:				
Dixie National Forest, Utah, near Cedar City.	4.4-5.1	4.8	2.4-2.7	2.6
Do.	4.4-4.7	4.6	2.4-3.0	2.7
Bryce Canyon National Park, Utah.	4.1-5.1	4.6	2.4-2.7	2.6
Do.	4.4-5.1	4.7	2.0-2.7	2.5
Roosevelt National Forest, Colo.	4.4-5.4	4.8	2.4-2.7	2.6
Harney National Forest, S. Dak., near Custer.	4.1-5.1	4.5	2.4-2.7	2.5
Do.	4.4-5.1	4.6	2.4-3.0	2.7
Total of 140 measurements, usual range, mean.	4.1-5.1	4.7	2.4-3.0	2.6
Territory where some of the beetles are atypical: <sup>2</sup>				
Elk Mountain, Carbon County, Wyo.	4.4-5.1	4.6	2.4-3.0	2.8
Medicine Bow National Forest, Wyo.	4.4-5.1	4.6	2.4-3.0	2.7
Total of 40 measurements, usual range, mean.	4.4-5.1	4.6	2.4-3.0	2.8
Total of 400 measurements, usual range, mean.	4.4-5.1	4.6	2.4-3.0	2.7

<sup>1</sup> In every case these figures are based on the measurements of 20 ascospores.<sup>2</sup> See footnote 4, p. 590.

## TEMPERATURE RELATIONS

*Ceratostomella montium* is adversely affected by high temperatures and low humidities. Twelve of the first sets of *Dendroctonus monticolae*-infested pine specimens collected between 1930 and 1933 produced no fungi when cultured, while from other sets *C. montium* was occasionally isolated. In 1935, 1936, and 1937, when specimens and cultures were held in incubators at temperatures of 12° to 17° C., the cultures of 18 sets of *D. monticolae*-infested wood or of adult beetles produced *C. montium*. Also, cultures made from specimens of *D. ponderosae*-infested wood or from adult beetles developed *C. montium* when held at 12° to 17°. The cultures grew and fruited best when held in an incubator at a temperature of 16°. At this temperature the beetles and larvae used as inocula remained alive for 2 weeks. The fungus grew well, however, when held in incubators at temperatures between 12° and 22°. When the temperature was raised to 27° the mycelium began to deteriorate and yeasts grew vigorously. According to a Weather Bureau map defining the climatic provinces in

the central Rocky Mountains, the mean temperature where these specimens were collected is approximately 50° F.<sup>6</sup>

### TECHNICAL DESCRIPTION

#### *Ceratostomella montium* n. sp.<sup>7</sup>

Young colonies with conidia white, changing to warm sepia and black, producing perithecia; young hyphae hyaline, 1.3 $\mu$  to 4 $\mu$  in diameter; old hyphae brown, septate, 4 $\mu$  to 8 $\mu$  in diameter; conidiophores single, hyaline, at first unbranched, later simple branched hyphae bearing conidia in clusters; conidia hyaline, clustered or solitary, appearing on hyphae and short conidiophores, globular, 4 $\mu$  to 5 $\mu$ , ovoid to clavate, 6.5 $\mu$  to 8 $\mu$  by 4 $\mu$  to 5 $\mu$ ; perithecia black, globose, slightly hirsute; height of base, range 175 $\mu$  to 448 $\mu$ , usual range 250 $\mu$  to 330 $\mu$ , mean 287 $\mu$ ; width of base, range 162 $\mu$  to 410 $\mu$ , usual range 238 $\mu$  to 320 $\mu$ , mean 278 $\mu$ ; neck longitudinally striate; length of neck 552 $\mu$  to 3,776 $\mu$ , usual range 1,141 $\mu$  to 1,902 $\mu$ , mean 1,527 $\mu$ ; mean width of neck at tip, 21 $\mu$ ; the ostiole without cilia; asci ephemeral, clavate or ellipsoid; ascospores 8, one-celled, hyaline, rectangular parallelipedal with square ends; length, range 3.7 $\mu$  to 5.8 $\mu$ , usual range 4.4 $\mu$  to 5.1 $\mu$ , mean 4.6 $\mu$ ; width, range 2.0 $\mu$  to 3.4 $\mu$ , usual range 2.4 $\mu$  to 3.0 $\mu$ , mean 2.7 $\mu$ .

On sapwood of *Pinus monticola*, *P. contorta latifolia*, and *P. flexilis* infested with *Dendroctonus monticolae*, and of *P. ponderosa*, *P. flexilis*, and *P. contorta latifolia* infested with *D. ponderosae*, in forests in the central Rocky Mountains, United States of America.

### DIAGNOSIS

#### *Ceratostomella montium* sp. nov.

Coloniis juvenilibus conidiferis albis tum brunneis-nigrescentibus cum peritheciis; hyphis hyalinis 1.3 $\mu$ -4 $\mu$  diam., deinde brunnescentibus 4 $\mu$ -8 $\mu$  diam.; conidiophoris hyalinis, simplicibus ramosis; conidiis hyalinis, in hyphis primo apparentibus, globosis 4 $\mu$ -5 $\mu$ , ovoideis vel clavatis 6.5 $\mu$ -8 $\mu$   $\times$  4 $\mu$ -5 $\mu$ , solitariis, deinde in massulis congregatis; peritheciis nigris, globosis, leniter hirsutis, 175 $\mu$ -448 $\mu$  altis, 162 $\mu$ -410 $\mu$  latis; rostellis 552 $\mu$ -3,776 $\mu$  longis, ostiolo carente filamentis; ascis evanidis, clavatis, octosporis, ascosporis hyalinis, formis rectangular parallelipedal, quadratis extremis, 3.7 $\mu$ -5.8 $\mu$   $\times$  2 $\mu$ -3.4 $\mu$ .

In ligno sapido *Pinus monticola*, *P. contorta latifolia*, *P. flexilis* *Dendroctono monticolae* infestato, et in ligno sapido *P. ponderosa*, *P. flexilis*, *P. contorta latifolia* *Dendroctono ponderosae* infestato.

Silvis in mediis Rocky Mountains, United States, America.

### YEASTS ASSOCIATED WITH CERATOSTOMELLA MONTIUM

From all of the inocula yeasts<sup>8</sup> developed first, and later *Ceratostomella montium* appeared, growing out from the yeast colonies (fig. 5, A). They were of the same two types of yeast associated with the bark beetles that previously had been studied for fungus associations (7, 15, 16). In the laboratory the yeasts endured and grew at higher temperatures than *C. montium*.

Most of the yeasts belonged to species of *Monilia*. The term *Monilia* here means, according to Guilliermond (5), Henrici (6), and Stovall and Bubolz (18), a non-spore-forming, or anascosporous,

<sup>6</sup>In 1903 Von Schrenk (20) described a blue stain fungus, *Ceratostomella pilifera* (Fr.) Wint. which he isolated from ponderosa pines infested with *Dendroctonus ponderosae* collected in the Black Hills Forest Reserve, S. Dak. (now the Harney National Forest), but he was unsuccessful in isolating a blue-staining fungus from the beetles. The high temperatures in the laboratory at the Missouri Botanical Garden, St. Louis, Mo., where the cultures were made, may have suppressed the growth of *C. montium* and encouraged that of *C. pilifera*. Scheffer and Lindgren (17) have shown that geographical strains of *C. pilifera* differ measurably in their reaction to temperature. The maximum rate of growth of several strains has been found to occur at 28° to 29° C., while growth is inhibited at 35°.

<sup>7</sup>Since this paper was prepared, the name *Ceratostomella montium* Rumbold has been used by Vinje (19), to whom the writer supplied cultures and who described the development of the perithecia.

<sup>8</sup>These yeasts were cultured and identified by Eugene C. Holst, formerly student assistant, Division of Forest Pathology, now assistant bacteriologist, Bureau of Entomology and Plant Quarantine.



FIGURE 5.— A, Culture on malt agar of *Ceratostomella montium* and anascosporous yeasts from an adult *Dendroctonus ponderosae*: a, Black mycelium; b, white glistening yeast; c, black perithecia growing on surface of yeast. About  $\times 10$ . B, A 9-day-old culture of anascosporous yeast isolated from an adult *D. ponderosae*.  $\times 12$ .

budding yeast, which may also exist in a mycelial form (fig. 5, B) with the possibility of conidia being borne on the hyphae.

These anascosporous yeasts are mostly *Mycocandida* and *Mycotoruloides* types, defined by Langeron and Talice (10) and Lodder (12). One of the types isolated from *Dendroctonus monticolae* collected in the Metaline Falls, Wash., region was found to resemble a type isolated from the insect *D. piceaperda* Hopk. collected in Canada.<sup>9</sup>

In addition to the usual anascosporous mycelium-forming yeasts, *Zygosaccharomyces pini* Holst (7) was isolated from seven *Dendroctonus ponderosae* beetles collected in Harney National Forest, S. Dak.

The different species of yeasts grew on sterilized pine on which the blue stain fungus did not grow. They have a stimulating effect on the staining fungus, causing it to grow more vigorously on agar and to fruit more quickly than the pure cultures of *Ceratostomella montium*. They are frequently found growing up the outside of the necks of the perithecia, so that when ascospores are ejected from the ostioles, yeast is mixed with them.

## DISCUSSION

*Ceratostomella montium* is probably too sensitive to environmental influences

<sup>9</sup> HOLST, E. C. YEASTS ASSOCIATED WITH SEVERAL SPECIES OF BEETLES. Prog. Rpt., Div. Forest Path. U. S. Bur. Plant Indus., July 1, 1934. [Unpublished manuscript.]

to be able to spread far from the mountain forests. In this it differs from *C. ips*, which can grow in a wide range of temperatures ( $10^{\circ}$  to  $35.5^{\circ}$  C.) and can infect freshly sawed boards in southern lumberyards. While the mycelium of *C. montium* can permeate malt agar for  $1\frac{1}{2}$  inches below its surface, its perithecia develop only on the surface of the medium. Perithecia of *C. ips* are frequently found beneath the agar surface. Though the perithecia and ascospores of *C. montium* are larger than those of *C. ips*, whose height of base of perithecium averages  $206\mu$ , width  $198\mu$ , and length of neck  $1,273\mu$ , this fungus looks like *C. ips*, in many respects, such as the lack of cilia at the ostiole, the shape of the ascospore, rectangular parallelepiped with square ends, appearance of germinating ascospores, and formation and shape of conidia. This fungus does not, however, produce either the well-developed conidiophores or the fascicles of conidiophores which develop in cultures of *C. piceaperda* and *C. ips*. Its conidiophores are simple, and in this phase of growth it resembles *C. pseudotsugae* Rumbold (15.)

An association of the same blue stain fungus with more than one species of bark beetle is not unusual. *Ceratostomella pini* Münch is associated with *Dendroctonus frontalis* Zimm. on the Atlantic coast and with *D. brevicornis* Lec. on the Pacific coast. *C. ips* is associated with different species of *Ips* on the Atlantic and Pacific coasts and in the Central States (11). Whether *C. montium* is associated with *D. monticolae* found in the forests in the Sierras on the Pacific coast or in Canada or with *D. ponderosae* in the Kaibab National Forest or in Mexico is not known.

The two types of yeast, *Zygosaccharomyces pini* and the anascoporous yeasts, that were found regularly associated with *Dendroctonus monticolae* and *D. ponderosae*, are those found with other species of bark beetles. They appear to be widespread in the forests in this country. They are regarded as of secondary importance in causing the death of insect-infested pines. Experiments of Nelson and Beal (13) showed that blue stain fungi inoculated into pine trees killed them without the aid of bark beetles or yeast. Similar experiments by Bramble and Holst (2) gave the same result. They found that when inoculated into pines the yeast *Zygosaccharomyces pini* showed no evidence of its pathogenicity. Holst (8) found that the life of the bark beetle *D. frontalis* was not dependent on yeast, as he raised sterile larvae and an adult from surface-sterilized eggs.

It was noticed that when there was an epidemic of *Dendroctonus monticolae* and *D. ponderosae* in the forest the specimens sent in, whether insects or the bark or sapwood of infested pines, developed *Ceratostomella montium* and yeast in almost pure form. When the beetle infestation in the forest was light, however, the *C. montium* cultures isolated grew slowly and other fungi also were isolated. The close association of beetle and fungus could be seen in the original test-tube cultures when adults or larvae were the inocula. They developed vigorous cultures of *C. montium*, with large perithecia growing on the bodies of insects and larvae.

#### SUMMARY

The bark beetle *Dendroctonus monticolae* infests mountain pines in the central Rocky Mountain forests, extending from the Canadian

boundary south to the Targhee National Forest in eastern Idaho to the Wyoming and Washakie National Forests in southwestern Wyoming and the Wasatch and Uinta National Forests in northeastern Utah. It carries with it in its attacks on the pines a blue stain fungus, *Ceratostomella montium*, a *Ceratostomella* not previously described. This fungus is disseminated also by the bark beetle *D. ponderosae* when it infests pines in mountain forests extending from Dixie National Forest in southwestern Utah, Bryce Canyon National Park in south-central Utah, and in forests extending northeast to the Roosevelt National Forest in northeastern Colorado, Medicine Bow National Forest in southeastern Wyoming, and Harney National Forest in southwestern South Dakota. It is not known whether this fungus is associated with the beetles in other parts of their range.

It is doubtful whether *Ceratostomella montium* will spread from the mountain forests, for in artificial culture this fungus does not tolerate high temperatures. It has been found to grow best at a temperature of 16° C., and grew well between 12° and 22°.

*Ceratostomella montium* grows beneath the bark of beetle-infested pines. At the time the insects are emerging, the long necks of the large perithecia grow into the galleries, where they appear like thin black wires. The fungus-infected sapwood usually is gray, shading to blackish green gray.

The beetles carry with them, besides the blue stain fungus, two types of yeast—an anascoporous mycelium-forming group and *Zygosaccharomyces pini*.

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# EFFECT OF TEMPERATURE DURING IRRADIATION ON THE X-RAY SENSITIVITY OF MAIZE SEED<sup>1</sup>

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## INTRODUCTION

Certain organisms in the resting phase respond to heavy X-ray dosages by dying soon after an apparently normal early growth. In the case of air-dry maize seeds this phenomenon, which has been called delayed death (1),<sup>2</sup> is characterized by death of the seedling following normal germination and development to a stage where the first leaf is partially exerted from the coleoptile. This behavior has been noted for all the common cereals and numerous dicotyledonous species. Comparable behavior has been observed in the fungus *Ustilago hordei*.<sup>3</sup> Sugiura (12) has reported a similar response for wheat seedlings, and Enzmann and Haskins (4) have noted delayed death for many garden seeds and also for the vinegar fly, *Drosophila*.

Tests for the possible destruction of growth substances indicate that auxin production is not directly interfered with by the irradiation used, although there is evidence that the transportation mechanism is restricted.<sup>4</sup> It is believed, therefore, that delayed death cannot be explained as a secondary reaction brought about by the lack of growth substances. A break-down in transportation, however, points to a disruption of cellular dynamics, of which the failure to transport growth substance is one manifestation.

A possible explanation of the partial growth but early death of seedlings from X-rayed seeds can be based on the profound derangement of the chromatin produced by X-rays. It has been shown by Stadler (10) and others that chromosomes are broken as the result of X-raying seeds and that these breaks lead to translocations, inversions, deletions, and fragmentations.

Whiting (13), experimenting with irradiated unfertilized eggs of *Haemaphysalis*, concluded that the death of eggs is correlated with their chromatin condition. She found that eggs in the first meiotic metaphase failed to hatch, that those in the latest prophase (diakinesis) hatched but failed to mature, and that those newly differentiated (preleptotene) had a mortality of 69.6 percent and a large percentage died as larvae. The younger oocytes with nurse and follicle cells (early prophase) are the most resistant with the exception of those in the late prophase with diffuse chromatin, which have the lowest mortality of all.

Stone (11) studied the effect of X-rays on the mitotic divisions in the root tips of *Crocus olivieri* and concluded as follows:

It appears likely that the sequence of events is as follows. X-ray treatment causes a relatively spontaneous physiological reaction, which is sufficient to suppress

<sup>1</sup> Received for publication January 24, 1941.

<sup>2</sup> Italic numbers in parentheses refer to Literature Cited, p. 618.

<sup>3</sup> RODENHISER, H. A., and MAXWELL, LOUIS R. [Unpublished manuscript.]

<sup>4</sup> DU BUY, H. G. [Unpublished manuscript.]

further division in resting nuclei but not able to stop the process of division already begun. This is in agreement with Strangeways and Hopwood,<sup>5</sup> but it is possible to go further and suggest that this change increases slowly in intensity, and when the intensity is at its highest acts in a similar way upon the resting nuclei, derived from divisions allowed to proceed normally after treatment. Activity is suspended for a certain period (depending upon the dose applied), and then the effect wears off and mitoses begin to appear. Many are abnormal, and it is clearly possible that these abnormalities may have been initiated during the post-mitotic as well as during the pre-mitotic resting stages following treatment.

Collins and Maxwell (1) report that in root-tip material of delayed-killed maize seedlings Longley found all mitoses abnormal in some degree, the abnormalities ranging from divisions with lagging chromosomes to divisions in which the chromatin was an undifferentiated amorphous mass. Such nuclear disorganizations produce unequal distribution of chromatin when cells divide. Thus, although a few cell generations are possible with unequally divided chromosomes eventually the loss of balance between nuclear elements prevents further division and growth ceases. This idea finds support in the fact that under normal conditions the cells of the lower leaves of maize have a life limited at most to a few weeks. In the absence of continuing cell generations the first leaves of uninjured seedlings presumably die simply because the cells are short-lived. When normal maize seedlings have their growing point above the first true leaf removed, this leaf may survive 3 weeks—a period not much longer than the time seedlings from X-rayed seeds may live.

Although delayed death is a consistent result obtained with air-dry maize seeds for dosages greater than 60 kr.,<sup>6</sup> this is not the case when dosages approach the delayed-death threshold. In very exceptional cases 100 percent delayed killing has been obtained with a dosage as low as 20 kr., while in other instances a high percentage survived at 35 kr. In all these experiments the X-ray dosage was under rigid control, having a maximum variation of 5 percent. The erratic results in seedling survival at the threshold dose undoubtedly may be ascribed to important uncontrolled factors operative after planting. Some progress has been made in determining the factors of environment that affect survival, but the results are not yet consistent enough to justify conclusions.

More certain results have been obtained with temperature treatments given the seeds during the time of irradiation. It has been shown (7) that when seeds are held at liquid-air temperature during irradiation their sensitivity to X-rays is reduced, as determined by the extent of seedling growth in the delayed-killed stage. Apparently those factors responsible for delayed death are diminished by exposure to liquid-air temperature during irradiation.

The present paper reports further experiments on the effect of the temperature of air-dry seeds during the time of irradiation on the growth of maize (*Zea mays* L.) seedlings. The temperatures investigated ranged from  $-187^{\circ}$  to  $66^{\circ}$  C.; X-ray dosages ranged from 35 to 45 kr.

#### EXPERIMENTAL METHODS

The X-ray set-up used for the irradiation has been described previously (7). The seeds were placed in a metal boat that floated on

<sup>5</sup> Citation to literature in Stone (11).

<sup>6</sup> Abbreviation of kiloroentgen; 1 kr. equals 1,000 roentgens.

liquid air for the  $-187^{\circ}$  C. temperature, in a thick mixture of carbon dioxide snow and alcohol for the  $-66^{\circ}$  temperature, in crushed ice and water for the  $0^{\circ}$  treatment, in water at room temperature for the room-temperature point, and in hot water for the  $50^{\circ}$  temperature. For the  $66^{\circ}$  temperature, an oven with temperature controls was used. Seed temperatures were determined by embedding a thermocouple in one of the seeds. Temperatures given are accurate within  $\pm 5^{\circ}$  for the liquid air flask arrangement and  $\pm 2^{\circ}$  for the oven. When the oven was used the seeds were lying on a sheet of paper; therefore the dosages given under these conditions were increased by about 5 percent to subject the seeds to the same dosage as that given in the liquid-air flask arrangement. Under the conditions of irradiation, i. e., with 45-kr. constant potential, tungsten anode, and no appreciable filtration, the amount of back-scattering was measured and was found to be about 8 percent. Dosage values are measured by an open air ionization chamber and are accurate within 5 percent. Exposure time ranged from  $3\frac{1}{2}$  to 5 hours.

The seed used throughout was Funk Yellow Dent drawn from the large, thoroughly mixed sample used in previously reported experiments. The lot from which the treated samples were drawn was stored at  $1^{\circ}$  to  $2^{\circ}$  C. In all the experiments reported here, except 1, the seedlings were grown in a greenhouse in metal or rubber flats. These flats accommodated 144 seedlings in 12 rows of 12. They were 1 foot square and  $4\frac{1}{2}$  inches deep. After the seed was sown, the flats were mounted on compound clinostats, where both the table and the individual flats rotated independently. Tests were made in randomized blocks, each flat containing 2 or more complete series. Where the experiment was of such a size that more than 1 clinostat was required, the choice of flats between clinostats was also random.

With the exception of the last four experiments described below the seedlings were grown in carefully mixed soil covered with a half-inch layer of water-washed sand. Usually the variance between flats exceeded that which might be attributed to chance in spite of meticulous care in distributing the sterilized soil among the flats, in compressing it before planting, and in watering before and during the course of the experiments. Accordingly various tests were conducted with sand and soil, with and without culture solutions. The least variance between flats was obtained with sand saturated with the culture solution given by Eaton (3). Consequently this system has been used for the last four experiments reported. The seeds were planted point down, at a depth of one-half inch in soil or in water-washed sterilized sand brought to a moisture content of about 25 percent. The flats were covered with impervious black paper until the seeds germinated. Then the paper was removed and the flats were brought up to saturation each morning. In all cases the seedlings were measured at least twice and in some instances five times, at intervals of 2 days. Failure to elongate between any two measurements indicated death. Most of the experiments were continued until all question of survival or demise was settled.

The variance was analyzed for each series of measurements for each experiment, and the errors given below are from generalized standard deviations for each experiment. As a rule the variance of blocks within flats and between clinostats never exceeded that expected

by chance, and in the last four experiments this was true of the variance between flats.

### RESULTS

The results are presented in chronological order. This is done to illustrate the reproducibility of the effects observed and also because the experiments lead from one to another.

#### EXPERIMENT 1, THREE-POINT LARGE-TEMPERATURE-VARIATION TESTS, APRIL 1939

Three lots of 288 seeds each were given a dosage of 35 kr. These were held at  $-187^{\circ}$  C., room temperature, and  $61^{\circ}$ , respectively, during the time of treatment. The seeds were planted on April 10, 1939, and the experiment was terminated on April 20, since by that date the seedlings from seeds X-rayed at room temperature were dying. By April 26 all the seedlings of the room-temperature series were dead. No final count of survivors was made, because some of the flats were broken down for photographing. A summary of the results obtained is given in table 1.

TABLE 1.—*Effect of seed temperature during X-raying (35 kr.) on size of seedlings, April 1939*

Seed temperature ( $^{\circ}$ C.) at exposure to X-rays	Total seedlings <sup>1</sup>		Mean height of seedlings	
	Apr. 17	Apr. 20	Apr. 17	Apr. 20
	Number	Number	Millimeters	Millimeters
$-187^{\circ}$ .....	254	253	19.30 $\pm$ 0.74	27.41 $\pm$ 1.53
21 (room).....	279	270	8.51 $\pm$ .71	6.44 $\pm$ 1.46
61.....	285	284	35.66 $\pm$ .70	81.82 $\pm$ 1.45

<sup>1</sup> 288 seeds in each treatment.

It is noticed that, in agreement with results previously reported (7), the sensitivity of seeds held at  $-187^{\circ}$  C. was definitely less than the sensitivity of seeds kept at room temperature. The seeds maintained at  $61^{\circ}$ , however, produced plants having the greatest height. Figure 1 shows a photograph of three plants typical of the three temperature groups. Chlorophyll deficiencies may be noted in the surviving plants.

To determine the effect of temperature alone, a duplicate set of 288 seeds was held at each of these temperatures for the same length of time as the X-rayed series ( $3\frac{1}{2}$  hours) and in the same containers and positions. These non-X-rayed seeds were planted April 13 in the same manner as those of the X-rayed series and were measured April 21. Each lot of seedlings from this non-X-rayed material was measured only once, and the results are shown in table 2.

TABLE 2.—*Effect of seed temperature without X-rays on size of seedlings, April 1939*

Seed temperature ( $^{\circ}$ C.) at exposure	Total seedlings <sup>1</sup>	Mean height of seedlings
	Number	Millimeters
$-187^{\circ}$ .....	235	103.08 $\pm$ 1.59
21 (room).....	280	140.96 $\pm$ 1.46
61.....	281	139.43 $\pm$ 1.46

<sup>1</sup> 288 seeds in each treatment.

It is seen that heating the seeds to 61°C. for 3½ hours had no effect on the size of the seedlings but that exposure to low temperature had a pronounced detrimental effect on both germination and size. From



FIGURE 1.—Typical plants selected from experiment 1 to illustrate temperature effects. Treatment temperatures during irradiation (35 kr.) were as follows: A, 21° C.; B, -187°; C, 61°.

table 1, it is evident that germination was low also for seeds X-rayed at -187°, but the plants which did appear grew definitely taller than those from seeds X-rayed at room temperature.

## EXPERIMENT 2, THREE-POINT SMALL-TEMPERATURE-VARIATION TESTS, MAY 1939

In experiment 2, 2 X-ray dosages (30 and 35 kr.), 3 temperatures (20°, 50°, and 60° C.), and 6 lots of 48 seeds each were used. The seeds were planted on May 17, 1939, and the seedlings were measured on May 22, 24, 26, and 29. A summary of the results is given in table 3. By May 26 delayed death from some treatments was evident, so the mean heights are given in table 3 for the measurements made on May 24. The number of surviving seedlings was determined May 29.

TABLE 3.—*Effect of seed temperature during X-raying on size of seedlings and number of survivors, May 1939*

X-ray dosage (kiloroentgens)	Seed temperature at exposure	Total seedlings <sup>1</sup>	Mean height of seedlings	Seedlings surviving
	°C.	Number	Millimeters	Number
30.....	20	46	8.67±0.93	10
	50	42	51.89±.97	42
	60	45	47.56±.94	45
35.....	20	47	8.15±.92	1
	50	47	37.40±.92	43
	60	48	34.06±.91	46

<sup>1</sup> 48 seeds in each treatment.

It is obvious that treatments at temperatures of 50° and 60° C. resulted in increased height and number of survivors as compared with room temperature and that the difference in X-ray dosage of 5 kr. produced a significant difference in seedling size at the two higher temperatures. The interaction of dosage with temperature was not significant, a result to be expected since the two X-ray dosages were not greatly different. Although in both X-ray treatments the seeds held at 50° gave larger seedlings than those held at 60°, the differences within X-ray dosages are not significant. The possibility is suggested in this experiment that the most effective temperature for resistance to the effects of X-rays is between 50° and 60°.

## EXPERIMENT 3, MOISTURE-SENSITIVITY TESTS, JUNE 1939

Since raising the temperature of seeds will reduce their moisture content, the next experiment in this series was designed to determine the effects of X-rays on seeds of low moisture content. For this purpose a sample of 288 seeds was dried in a vacuum oven at 70° C. until the moisture content had been reduced from 8 to 2 percent. One hundred and forty-four of these seeds were given an X-ray dose of 35 kr. at room temperature. The remaining 144 seeds were used as controls. There were also included in this experiment 144 stock seeds of 8-percent moisture subjected to 35 kr. of X-radiation at room temperature and an equal number not X-rayed. These four lots of seeds were planted on June 15, and the seedlings were measured on June 20, 21, 22, 23, and 26. The statistical treatment was confined to the measurement on June 22, at which date germination was complete and delayed death had not become apparent. The results are shown in table 4.

TABLE 4.—*Number and mean height of seedlings from oven-dried (2-percent moisture) and stock (8-percent moisture) seeds X-rayed and not X-rayed, June 1939*

Condition of seed	Treatment	Total seedlings <sup>1</sup>	Mean height of seedlings	Seedlings surviving
		<i>Number</i>	<i>Millimeters</i>	<i>Number</i>
Oven-dried (2-percent moisture) . . .	Not X-rayed . . . . .	123	126.33±1.06	123
	X-rayed (35 kr.) . . . . .	90	11.41±1.22	0
Stock (8-percent moisture) . . . . .	Not X-rayed . . . . .	123	125.49±1.06	123
	X-rayed (35 kr.) . . . . .	128	49.87±1.03	92

<sup>1</sup> Each lot of seeds treated totaled 144.

It will be noted that a reduction in the moisture content had no effect on seedling size when the seeds were not X-rayed, but when they were treated the deleterious effect of the drying was pronounced both with respect to survival and mean height of surviving plants. Insofar as this experiment can be considered typical, the beneficial effect of high temperature during irradiation cannot be ascribed to a reduction in moisture content.

## EXPERIMENT 4, TWO-POINT SMALL-TEMPERATURE-VARIATION TESTS, AUGUST AND SEPTEMBER 1939

In experiment 4 an X-ray dosage of 45 kr. and seed temperatures of 31° C. (room temperature) and 51° were used. The choice of 51° instead of 60° was made because in experiment 2 (table 3) the seeds treated at 60° showed the least sensitivity to X-radiation. The seeds were heated in the oven mentioned above. Two hundred seeds were X-rayed at each temperature. Of these, 144 from each lot were grown in the greenhouse and 48 in an air-conditioned room maintained at approximately 25°, with continuous illumination at 300 foot-candles (Mazda). The plantings were made on August 26, and measurements were made August 31 and September 2, 5, and 7. At the second measurement, germination was complete and no seedlings had died. The results are shown in table 5.

TABLE 5.—*Effect of X-ray treatment (45 kr.) of seeds <sup>1</sup> on mean height and number of surviving seedlings, August and September 1939*

Seed temperature (° C.) at exposure to X-rays	Location of plants	Total seedlings	Mean height of seedlings	Seedlings surviving <sup>2</sup>
		<i>Number</i>	<i>Millimeters</i>	<i>Number</i>
31 . . . . .	Greenhouse . . . . .	142	26.13±0.93	107
51 . . . . .		141	29.30±.93	78
31 . . . . .	Air-conditioned room . . . . .	47	25.43±1.62	—
51 . . . . .		47	31.38±1.62	—

<sup>1</sup> 200 seeds were X-rayed at each temperature; of these, 144 seeds from each lot were grown in the greenhouse and 48 in an air-conditioned room at approximately 25° C., with continuous illumination at 300 foot-candles.<sup>2</sup> The seedlings in the air-conditioned room could not be classified on a survival basis as could those in the greenhouse.

The differences in height of plants between the two treatment temperatures would be expected as the result of chance about once in 20 trials and therefore can hardly be considered significant. However, since both trials showed the same behavior and to about the same degree, somewhat more confidence may be placed in the results. At the fourth measurement, death had overtaken a large number of the



seedlings and, since the survivors were evidently capable of completing growth, the experiment was terminated.

For plants grown in the greenhouse, the  $\chi^2$  of the distribution of living and dead plants from the two seed treatments on the assumption that the two populations are random samples from the same lot, is 15.04; from this it is concluded that the seed temperature during irradiation was an important factor in determining whether the seedlings were to live or die. This confirms the beneficial effects derived from using high temperature during irradiation.

#### EXPERIMENT 5, SIX-POINT LARGE-TEMPERATURE-VARIATION TESTS, DECEMBER 1939

In experiment 5 an X-ray exposure of 45 kr. and temperatures during radiation of  $-187^\circ$ ,  $-66^\circ$ ,  $0^\circ$ ,  $27^\circ$ , and  $56^\circ$  C., were used and 1 lot was X-rayed at  $27^\circ$  and was then heated at  $60^\circ$  for the same length of time as the others, namely,  $4\frac{1}{2}$  hours. Two hundred seeds were treated at each temperature, and 192 of each of these were planted in 8 flats on December 4. The results are shown in table 6.

TABLE 6.—*Effect of seed temperature during X-raying (45 kr.) on number of survivors and on height of seedlings grown in the greenhouse, December 1939*<sup>1</sup>

Seed temperature ( $^\circ$ C.) at exposure to X-rays	Total seedlings	Mean height of seedlings <sup>2</sup>	Seedlings surviving
	Number	Millimeters	Number
$-187^\circ$	187	24.00	27
$-66^\circ$	184	18.00	0
$0^\circ$	187	14.96	0
$27^\circ$	190	14.53	0
60 after $27^\circ$	187	15.90	0
$56^\circ$	189	19.46	3

<sup>1</sup> 200 seeds were treated at each temperature; 192 seeds from each lot were planted in 8 flats December 4, 1939.

<sup>2</sup> Standard error equals  $\pm 0.47$ .

The possible differences between these treatments are given in table 7.

TABLE 7.—*Differences in mean height between the seedlings from various seed temperatures*<sup>1</sup>

[Based on data in table 6]

Seed temperature ( $^\circ$ C.) at exposure to X-ray	Differences between seedlings from indicated seed temperatures ( $^\circ$ C.)				
	$-187^\circ$	$-66^\circ$	$0^\circ$	$27^\circ$	60 after $27^\circ$
	Mm.	Mm.	Mm.	Mm.	Mm.
$-66^\circ$	5.91				
$0^\circ$	9.04	3.13			
$27^\circ$	9.47	3.56	0.43		
60 after $27^\circ$	8.10	2.19	-.94	-1.37	
$56^\circ$	4.54	-1.37	-4.50	-4.93	-3.56

<sup>1</sup> Minus sign indicates that the seedlings from the treatment shown at left were larger than those from the treatment designated by the column head.

As the standard error of a difference is  $\pm 0.67$ , it follows that differences to be significant must exceed 1.34 mm. Of the 15 differences, only 2 are below the level of significance, namely, the differences between seedlings from the seed temperatures  $0^\circ$  and  $27^\circ$  C., and

between seedlings from seeds X-rayed at 0° and from seeds given a heat treatment of 60° after being X-rayed at 27°. There are 2 other differences just over the border line of significance, namely, those between seedlings from seeds X-rayed at 27° and from seeds given a 60° heat treatment after being X-rayed at 27° and between seedlings from seed temperatures 56° and -66°.

From this experiment it must be concluded that the most sensitive seed temperature for the action of X-rays falls somewhere within the range of 0° to 27° C. In agreement with the results of previous experiments it was found that temperatures either above or below this point were effective in affording a degree of protection from the action of X-rays.

When the seedling flats of this experiment were broken down, it was observed that the length of the primary root varied with the seed treatment. Plants from two of the flats were lost before this observation could be put to the test, but the primary roots of the plants from the remaining six flats were measured, and the results are shown in table 8.

TABLE 8.—*Effect of seed temperature during X-raying (45 kr.) on length of primary roots*

Seed temperature (° C.) at exposure to X-rays	Total seedlings	Mean root length <sup>1</sup>	Seed temperature (° C.) at exposure to X-rays	Total seedlings	Mean root length <sup>1</sup>
	<i>Number</i>	<i>Millimeters</i>		<i>Number</i>	<i>Millimeters</i>
-187 .....	137	51.91	27 .....	140	37.28
-66 .....	136	41.37	60 after 27 .....	139	39.53
0 .....	139	37.44	56 .....	140	43.60

<sup>1</sup> Standard error equals  $\pm 1.05$ .

In general, these root data support the measurements of seedling height although the errors are greater, partly owing to the reduction in number. Possible differences are given in table 9.

TABLE 9.—*Differences in length of primary root between seedlings from various seed temperatures*

[Based on data in table 8]

Seed temperature (° C.) at exposure to X-rays	Differences <sup>1</sup> between seedlings from indicated seed temperatures (° C.)				
	-187	-66	0	27	56
	<i>Millimeters</i>	<i>Millimeters</i>	<i>Millimeters</i>	<i>Millimeters</i>	<i>Millimeters</i>
-66 .....	10.54				
0 .....	14.47	3.93			
27 .....	14.63	4.09	0.16		
56 .....	8.31	-2.23	-6.16	-6.32	
60 after 27 .....	12.38	1.84	-2.09	-2.25	4.07

<sup>1</sup> Minus sign indicates that the roots of seedlings from the treatment shown at left were longer than those from the treatment designated in the column head.

The standard error of a difference is  $\pm 1.48$ , so that to be considered significant differences must exceed 2.96. Of the 15 differences, 5 fall below the level of significance.

## EXPERIMENT 6, SIX-POINT LARGE-TEMPERATURE-VARIATION TESTS, JANUARY 1940

This experiment was essentially a repetition of the one just discussed. One hundred and ninety-two seeds at each temperature were planted in 8 flats January 15, and measurements were made on January 22, 24, and 26. During this period the sky was overcast, permitting a fair control of greenhouse temperatures but also resulting in a greatly reduced illumination which was accentuated by snow that covered the roof for 2 days. At the time of the first measurement there was a significant lag in germination of the seeds treated at 50° C. and a depressed growth at this temperature. By the second measurement, 2 days later, the germination had increased though it was still below average. At the same time the plants increased in size, growing more rapidly than those from any of the other treatments. This difference persisted to the close of the experiment and remains unexplained. Since the treatments were randomized in 8 flats on 2 clinostats and the data were consistent, the relatively poor and greatly delayed germination of the seeds treated at 50° C. cannot be attributed to the growing conditions. It is equally certain that there was no aberration in handling during exposure to X-rays. For the dosages given, only 100 seeds can be X-rayed at a time, so that each of these lots of 192 seeds was made up of two separate exposures. These lots were kept separate in planting, and the analysis of variance shows that the paired exposures were as nearly alike as could be expected from random sampling.

Delay in germination had the effect of placing these 50° C. seedlings in an environment somewhat different from those of the other treatments. But since the greenhouse temperatures were fairly uniform, the chief difference was in the amount of light the plants received.

By the third measurement, on January 26, delayed death was apparent in many of the treatments. The data are given in table 10; errors were calculated for only the second measurement.

TABLE 10.—*Effect of seed temperature during X-raying on size of seedlings and number of survivors, January 1940*

Treatment		Total seedlings			Mean height of seedlings			Seedlings surviving	
Seed temperature (° C.) at exposure to X-rays	X-ray dosage	Jan. 22	Jan. 24	Jan. 26 <sup>1</sup>	Jan. 22	Jan. 24 <sup>2</sup>	Jan. 26	Jan. 29	Jan. 31
	Kr.	No.	No.	No.	Mm.	Mm.	Mm.	No.	No.
—187.....	35	192	192	191	12.95	16.89	17.30	45	18
—62.....	35	186	186	186	12.86	16.51	16.72	25	15
0.....	35	187	187	187	12.01	15.39	15.53	21	5
27.....	35	185	185	185	12.29	16.13	16.36	15	5
50.....	35	138	173	177	11.15	20.29	24.88	146	94
66.....	36.7	185	188	188	14.45	22.30	23.93	27	12

<sup>1</sup> One plant was broken in measuring.

<sup>2</sup> Standard error equals  $\pm 0.55$ .

## EXPERIMENT 7, SIX-POINT LARGE-TEMPERATURE-VARIATION TESTS, MARCH 1940

In experiment 7 the temperatures duplicated those of experiment 6, but the X-ray dosage was reduced to 32.7 kr. except for the seeds treated at 66° C., in which case the dosage was 34.3 kr. This difference was applied to counteract the effect of back-scattering from the container used at the lower temperatures. One hundred and

ninety-two seeds from each of these treatments were planted in eight flats February 27, and measurements were made of the seedlings on March 4, 6, and 8. The measurements are given in table 11. Errors were calculated for only the last two measurements, when germination was complete. Delayed death was evident at the third measurement, and the number of living plants was recorded March 14.

TABLE 11.—*Effect of seed temperature during X-raying on size of seedlings and number of survivors, March 1940*

Treatment		Total seedlings			Mean height of seedlings			Seedlings surviving
Seed temperature (° C.) at exposure to X-rays	X-ray dosage	Mar. 4	Mar. 6	Mar. 8	Mar. 4	Mar. 6 <sup>1</sup>	Mar. 8 <sup>2</sup>	Mar. 14
	Kr.	No.	No.	No.	Mm.	Mm.	Mm.	No.
—187	32.7	186	188	188	14.52	16.77	16.97	0
—62	32.7	187	188	188	14.32	16.09	16.02	0
0	32.7	187	188	188	13.99	15.43	15.36	0
25	32.7	189	189	189	13.65	15.31	15.07	0
50	32.7	181	184	184	19.69	28.93	31.99	50
66	34.3	187	187	187	18.58	25.25	26.37	17

<sup>1</sup> Differences to be significant must exceed 1.66 mm.

<sup>2</sup> Differences to be significant must exceed 1.78 mm.

The seedlings from the 50° and 66° C. treatments were significantly larger than those from the lower temperatures. In this experiment, as in experiment 2, the seeds treated at 66° produced smaller seedlings than those treated at 50°. The difference in size of seedlings between the 50° and 66° lots may be the result of an overcorrection for back-scattering. However, it corresponds to that found in experiment 2, where the X-ray dosages were not modified to correct for back-scattering.

As in experiment 6, the seeds treated at 50° C. were slower in germinating and had a lower percentage of germination than those of the other treatments. In this experiment, however, it cannot be demonstrated that this peculiar behavior of seeds subjected to X-raying at 50° is other than a caprice of chance.

It is worthy of observation that although the plants in this experiment attained a size comparable to those in experiment 6, indicating closely similar growing conditions, the only surviving seedlings were found in the two high-temperature treatments.

#### EXPERIMENT 8, SMALL-TEMPERATURE-VARIATION TESTS, MARCH 1940

For the foregoing experiments the relative biological response was found to be reproducible within the temperature range —187° to approximately 50° C.; however, some uncertainty was found to exist for the higher temperatures. In experiment 2 the X-ray sensitivity appears slightly less for the 60° value than for the 50° point; in this case a hot-water bath was used for heating the seeds. However, as reported in experiments 6 and 7, the seeds kept at 66° showed greater injury than those held at 50°. In these later experiments, the temperature-control oven was used for the 66° value while the hot-water bath was used for the 50° point. Seeds in the oven rested on a thin paper support with practically no back-scattering, while the

seeds in the water bath received the back-scattering arising from the metal boat in which they were placed. Since tests made with the open ionization chamber showed that the amount of back-scattering from the metal boat was about 8 percent of the primary beam intensity, the dosage given to the seeds in the oven was increased by about 5 percent to compensate for the absence of back-scattering. The seeds were lying on the metal support with their germ sides up; thus a certain amount of the back-scattered radiation was probably absorbed in the endosperm so that the 5-percent factor may be an overcorrection, which would explain the apparent increase of X-ray sensitivity at 66°.

In order to test further the variations in sensitivity at these high temperatures, the oven was used for both the 50° and 66° C. treatments. By this means differences in back-scattering were avoided and the temperatures were more accurately controlled. Also, lots of seed were included to determine the effect of a dosage variation of about 5 percent. Three lots of 192 seeds each were treated, 2 lots at 66°, 1 of which was irradiated at a dosage of 35 kr., and 1 at a dosage of 36.7 kr. The third lot was treated at 51° with a dosage of 35 kr. The 3 lots of seeds were planted in the greenhouse on March 8 in 4 flats in randomized 3-row blocks. Measurements were made on March 14, 16, and 18, but only the results of the last measurement, at which time germination was complete and delayed death had not become apparent, are shown in table 12. The number of living plants was recorded March 23.

TABLE 12.—*Effect of seed temperature during X-raying on size of seedlings and number of survivors March 1940*

Treatment		Total seedlings <sup>1</sup>	Mean height of seedlings <sup>2</sup>	Seedlings living Mar. 23
Seed temperature (° C.) at exposure to X-rays	X-ray dosage			
	Kr.	Number	Millimeters	Number
51.....	35	189	26.97	32
66.....	35	184	29.34	63
66.....	36.7	183	25.04	36

<sup>1</sup> 192 seeds in each treatment.

<sup>2</sup> Standard error equals  $\pm 0.98$ .

In experiment 8, seeds X-rayed at 35 kr. showed a lower sensitivity at 66° than at 51° C. The experiment, however, was not efficient enough to establish differences of less than 10 percent in mean size. Limiting the comparison to the two temperatures having 35 kr. irradiation, it is seen that 66° has not exceeded the optimum for reducing sensitivity to X-radiation. Although the difference in mean height (2.37 mm.) is not statistically significant, the fact that there were twice as many survivors at the higher temperature may be considered strong evidence that the differences in seedling height are ascribable to the temperatures. The increase of 1.7 kr. of radiation produced a statistically significant reduction in seedling height.

#### DISCUSSION

The data on the percentages of survivors and on mean heights given in experiments 5, 6, and 7 can be consolidated to give values

showing more clearly the temperature effects within the range  $-187^{\circ}$  to about  $50^{\circ}$  C. In figure 2 is shown the percentage survival as a function of the temperature and in figure 3 are shown the mean

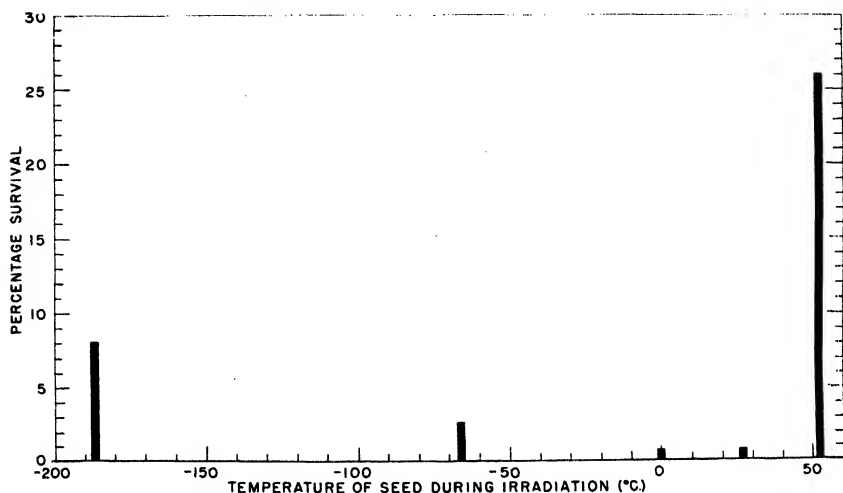


FIGURE 2.—Percentage survival as a function of seed temperature during irradiation (experiments 5, 6, and 7).

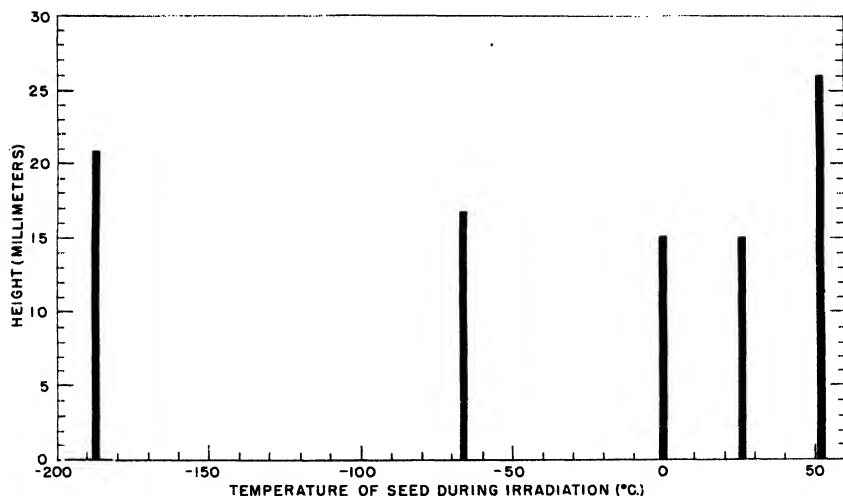


FIGURE 3.—Mean heights of plants as a function of seed temperature during irradiation (experiments 5, 6, and 7).

heights. The greatest survival is obtained at about  $50^{\circ}$ , and the lowest survival in the  $0^{\circ}$  to room-temperature range. In deriving the means it was necessary to weight each mean proportional to the mean height of its particular group. These diagrams suggest the possibility that quite different factors are influencing X-ray sensitivity. One set appears to predominate below about  $27^{\circ}$ , where the relation-

ship of temperature and X-ray sensitivity is essentially linear. Another set, which greatly reduces X-ray sensitivity, comes into operation above about 27°. The 66° value was not included because of the uncertainty mentioned above concerning the back-scattered radiation. However, on the basis of the data obtained in experiments 2 and 8, where no back-scattering factor entered, the 66° heat treatment resulted in a further reduction in the X-ray sensitivity.

The experiments described above show repeatedly and in a consistent manner that the X-ray sensitivity of maize seeds depends upon the temperature during the time of irradiation. It has also been demonstrated that the X-ray sensitivity is greatest in the range of room temperature to 0° C. An increase of temperature to 50°, or a decrease of temperature to -187° will in either case result in a lowering of the X-ray sensitivity.

A purely physical interpretation (6) of the mechanism responsible for delayed death showed, on the basis of the so-called "hit" theory, that the seed did not contain a single sensitive volume of dimensions required by the theory. It is possible, however, that the seed embryo contains several rather widely separated sensitive volumes, each one of which must suffer a number of hits by primary or secondary electrons to produce delayed death. Death of the plant may result, therefore, from an interaction between the sensitive volumes and the remainder of the material, as well as from interactions existing between themselves. Temperature alone obviously will not change the rate of quanta absorption or the number of hits per unit volume; however, the transportation mechanisms between various portions of the seed will be temperature-dependent. The observed decreased sensitivity at the low temperature may be due to a slowing up of the cell mechanism for dissemination of energy received from the primary electrons. This would result in a greater localization of the absorbed energy so that the activated sensitive volumes would be less effective in killing the plant. Although a theory of this kind may hold as an explanation of the reduced sensitivity at low temperatures, it fails to explain the response observed at high temperatures.

A conclusion that the ultimate cause of delayed death is to be found in the derangement of chromosomes with consequent breakdown in mitosis restricts the factors contributing to sensitivity to those which in some manner will affect the behavior of chromosomes. In the present experiments these factors must be present during exposure to X-rays. In resting seeds where no cell divisions are taking place, the chromosomes are not found as separate bodies and, although the chromatin appears as disconnected dots, these dots may be organized in an extended thread form. In this condition the chromosomes are subject to breakage. If the cause of seedling demise is due to physical injury of the chromosomes, then the external factors which reduce sensitivity to X-rays must operate in such a way as to protect the chromatin organization from these injuries or to heal the injuries after they occur.

Other investigators dealing with temperature-dependent sensitivities have attributed the reduced sensitivity at high temperatures to recovery. For example, Saks and Enzmann (8) investigated the effect of temperatures within the range 3° to 38° C. on chromosome breakage in *Tradescantia*. In their work flower buds were X-rayed at several temperatures and a cytological examination was made of the

chromosomes. It was found that with increase in temperature there was an apparent decrease in broken chromosomes. They attributed their failure to observe breakages at the higher temperatures to recovery. They assumed that breakages took place equally at all temperatures but at the higher temperatures the broken ends reunited.

Fabergé (5), also working with *Tradescantia*, has essentially confirmed the results of Saks and Enzmann by observing a reduction in the number of chromosome breakages when flower buds were X-rayed at 30° as compared with 15° C.

Spear (9) found that in animal-tissue cultures exposed to gamma rays at 37° and at 1° C. the reduction in mitosis was identical, but that recovery required a much longer time when cultures were irradiated at the lower temperature.

Cook (2), on the other hand, obtained greater recovery of one-cell *Ascaris* eggs from 5 kr. by holding them at 5° C. after treatment than at 25°. Delay in cleavage, however, was found to be temperature-independent. These recovery effects in tissue culture can hardly be applied to dry seeds, where there is no active cell division. It seems more likely that chromosome breakages are not the most important factor in the present experiments, since no obvious mechanism is apparent for reuniting the ends in the resting seeds.

No attempt has been made in the current experiments to establish the regularity of chromosome behavior as the result of high or low treatment temperatures in all the plants surviving these dosages. However, pollen mother cells of several such plants have been examined by Longley and found to be normal. Other plants have been transplanted and grown to maturity in the field without showing abnormal behavior. They produced viable pollen without excessive quantities of sterile grains, and they matured seed when self-pollinated and functioned as male parents on non-X-rayed plants. This behavior is fairly satisfactory evidence that these plants, and presumably, therefore, all the seedlings that survived, had normal cytological development. The only visible evidence of abnormality was a very fine striation in the leaves, found invariably on all the seedlings from X-rayed seed in these experiments.

Apparently the temperatures to be effective must prevail during irradiation. The single observation reported here (experiment 5) where the X-rayed seeds were subjected to high temperature following irradiation showed no significant beneficial effect of postirradiation temperature. Furthermore, when seeds were subjected to high temperature before irradiation (experiment 3) they were clearly more, rather than less, sensitive to X-rays. These results with anteirradiation and postirradiation temperatures are admittedly limited and are noted here as merely suggestive. Their bearing on the subject of the nature of X-ray injury can await a more adequate demonstration that temperatures at other than the irradiation period are ineffective. Experiments are now in progress to test this question.

#### SUMMARY

Certain organisms X-rayed in the resting stage respond to heavy dosages by dying soon after an apparently normal early growth. In the case of air-dry maize seeds this phenomenon, which has been called delayed death, is characterized by the death of the seedling after normal germination. Repeated experiments show in a consistent



manner that the X-ray sensitivity of air-dry (8-percent moisture) seeds of maize depends upon their temperature during the time of irradiation.

The temperatures used ranged from  $-187^{\circ}$  to  $66^{\circ}$  C. and the X-ray dosages from 30 to 45 kr. Heights of plants and the survival ratios were used for determining X-ray sensitivity.

It was found that maximum sensitivity occurred for the temperature range of  $0^{\circ}$  C. to room temperature. Either an increase or a decrease of the seed temperature from these values resulted in a reduction of the X-ray sensitivity.

X-ray dosages of 30 to 45 kr. are known to cause derangements of the chromosomes, but since the material used in these experiments was in the resting stage it is believed that the observed decrease in sensitivity at the extreme temperatures cannot be explained as recovery from injury to the chromosomes. Further questions concerning the interpretation of these results are discussed.

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# PRODUCTION OF HEAT AND OVULATION IN THE ANESTROUS EWE<sup>1</sup>

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## INTRODUCTION

In most sections of the United States the ewes of most breeds of sheep will breed only during the fall and winter months. Because of this fact, the majority of lambs are born in the spring and marketed in the fall. Lambs going to market during the spring and early summer generally bring a higher price, but for their production the ewes must be bred during the spring months. A sure but economical means of causing ewes to breed at this season would be of decided value to the sheep industry. Experimental attempts to produce heat and ovulation in the anestrous ewe by injections of endocrine substances are reported in this article.

Ewes may show signs of estrus without ovulating. It is necessary, therefore, to have some means of determining whether ovulation occurs. An operation permitting inspection of the ovaries has been employed by some investigators, but this procedure has very evident disadvantages. Suggestions have been made that ovulation could be detected by changes in the type of vaginal secretion, and a review of the vaginal smear records of the treated ewes has, therefore, been included in the study.

## REVIEW OF LITERATURE

Cole and Miller (3)<sup>2</sup> and McKenzie and Terrill (6) brought ewes into estrus during anestrus by injections of estrongenetic substances. The former used estrogenic hormone from mare urine. McKenzie and Terrill injected Progynon-B and were most successful with the use of 900 to 1,500 rat units.

The induction of ovulation accompanied by estrus has been reported by Cole and Miller (2, 3). The gonadotropic hormone of pregnant-mare serum was given in doses of 100 rat units spaced at 17-day intervals. Simultaneous injections of pregnant-mare serum and estrogen commonly resulted in estrus without ovulation. Injections of pregnant-mare serum followed by injections of estrogen 2 days later commonly resulted in ovulation without estrus.

McKenzie and Terrill (6) obtained ovulation without estrus in 5 of 11 anestrous ewes injected with 300 to 400 rat units of pregnant-mare serum.

Casida (1) has induced ovulation in 3- to 5-month-old lambs by injecting, subcutaneously, small doses of a pituitary extract, prepared as a partially purified follicle-stimulator.

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<sup>2</sup> Italic numbers in parentheses refer to Literature Cited, p. 625.

Changes in the vaginal smear have been reported by Hawkins and Darlow (5), Grant (4), Cole and Miller (3), Polovzeva and Fomenko (7), Richter and Rittau (8), and McKenzie and Terrill (6). The findings are not entirely in accord. Grant (4) and Richter and Rittau (8) doubt the reliability of the vaginal smear as an indicator of the stages of the estrual cycle. Cole and Miller (3), however, believe that the presence of large quantities of transparent, watery mucus characterizes early estrus; and a copious, dry, cheesy smear consisting chiefly of large squamous epithelial cells characterizes metestrus. Polovzeva and Fomenko (7) reported that the vaginal smear was a reliable indicator of ovulation, ovulation usually occurring after the appearance of cornified cells and before the appearance of leucocytes in the vaginal smear.

## EXPERIMENTAL PROCEDURE

### EXPERIMENTAL ANIMALS

The sheep used in this study included 30 yearling ewes, which were selected from two groups of feeder lambs, near Madison, Wis., in the spring of 1938. These ewes were of western origin (predominantly fine wool) and averaged about 85 pounds in weight at the time of purchase. They were kept in a barn during the entire experimental period, except for brief exercise periods when they were turned into a dry lot. Alfalfa hay consumed ad libitum was the sole feed during the study.

### VAGINAL SMEARS

Vaginal smears were taken from each ewe daily, except for a short period during the summer. In making these smears a speculum, made of 12-mm. glass tubing approximately 22 cm. long, and an 8-mm. glass rod, about 12 cm. longer than the speculum, were used. The ewe to be smeared was placed in a stanchion, the glass speculum inserted into the vagina and pushed forward and upward until the cervix was reached. The rod was then inserted through the speculum, the speculum withdrawn 1 to 2 inches, and the smear taken by rotating the inserted end of the rod on the exposed surface of the anterior vagina. The rod and speculum were removed after the upper end of the rod had been pulled back into the speculum to prevent contamination from any other region of the vagina at the time of withdrawal.

The consistency of the mucus found on the rod was noted and recorded as "thin," "thick," or "cheesy." If no mucus was present the smear was recorded as "clear." The smeared end of the rod was then rotated in a drop of water or eosin solution on a slide and this slide was examined under the low-power objective of a microscope. No actual counts of the various cell types found were made, but estimates of the numbers of the large, round, nucleated epithelial cells, the cornified epithelial cells, or "scales," and leucocytes were made. Each type of cell was given a value of "none," "very few," "few," "several," "many," or "very many."

### INDUCTION OF HEAT AND OVULATION

All the ewes were given endocrine injections to produce heat or heat and ovulation. Small groups, commonly four, were injected at different times during May, June, and July. All the ewes were

"teased" daily with aproned rams to determine if any were in heat (receptive to the male). The uninjected individuals at any treatment period thus served as controls for the appearance of heat in similar but untreated ewes. At the end of the period of experimental treatment, the ewes were spayed and the ovarian response to the treatment was checked at the time of removal of the ovaries.

#### ENDOCRINE SUBSTANCES USED

Progynon-B (Schering Corporation) is the benzoic-acid ester of dihydrofollicular hormone. This material was injected subcutaneously, commonly in the shoulder region. The pregnant-mare serum used was prepared by acetone precipitation and desiccation.<sup>3</sup> Immature-rat-ovary assays gave average weights of 36 mg. (4 rats) and 126 mg. (4 rats) from injections of 25 and 50 mg., respectively, of the preparation. Twenty-five mg. were then assumed to be equal to 1 rat unit. The material was injected subcutaneously as an aqueous suspension into the shoulder and flank regions. Sheep anterior pituitary extract was obtained from the zoological laboratory of the University of Wisconsin and was prepared from acetone-desiccated powder by extracting three times with water—ratio of 1 gm. of powder to 10 cc. of water. The aqueous extract was precipitated with acetone, recovered by centrifuging, and dried with acetone. Assays made by the zoological laboratory gave average ovarian weights as follows: 400 milligram-equivalents of the desiccated gland (12 rats), 115 mg.; 200 milligram-equivalents (12 rats), 91 mg.; 100 milligrams-equivalents (15 rats), 65 mg.; and 50 milligram-equivalents (12 rats), 41 mg.

TABLE 1.—Results of injections of endocrine substances upon the production of heat and ovulation

Number of ewes	Treatment <sup>1</sup>	Ewes showing heat	Ewes ovulating	Average number of ovulations in ewes that ovulated
8	Single injection of 100 rat units of pregnant-mare serum	0	7	1.56
4	100 rat units of pregnant-mare serum and 1,000 rat units of Progynon-B simultaneously.	1	2	1.00
2	100 rat units of pregnant-mare serum followed on the 1st, 2d, and 3d days by 2 injections of 500 rat units and 1 injection of 1,000 rat units of Progynon-B.	0	1	1.00
1	100 rat units of pregnant-mare serum followed on the 3d and 4th days by 2 injections of 1,000 rat units of Progynon-B.	0	0	0
1	100 rat units of pregnant-mare serum followed on the 3d day by a single injection of 1,000 rat units of Progynon-B.	2	0	0
3	1,000 rat units of Progynon-B in single injections	2	0	0
3	2,000 rat units of Progynon-B in single injections	1	0	0
2	2,000 rat units of Progynon-B in 3 successive daily injections of 500, 500, and 1,000 rat units.	0	4	6.25
4	2.5 gram-equivalents of sheep anterior pituitary extract given in 4 daily 0.5 gram-equivalent subcutaneous injections, followed by 0.5 gram-equivalent intravenous injection on the 5th day.	0	1	1.00
1	100 rat units of pregnant-mare serum and 1 gram-equivalent sheep anterior pituitary extract simultaneously.	0	1	3.00
1	100 rat units of pregnant-mare serum, 1 gram-equivalent sheep anterior pituitary extract, and 1,000 rat units of Progynon-B simultaneously.			

<sup>1</sup> Unless otherwise stated, injections were made subcutaneously.

<sup>3</sup> Grateful acknowledgment is made to Dr. W. H. McShan for the preparation of the pregnant-mare serum and the sheep anterior pituitary extract, and to Dr. R. K. Meyer for the assay of these materials.

## RESULTS

Ovulation was produced in seven of the eight ewes receiving 100 rat units of pregnant-mare serum (table 1). Five of the ewes had single ovulations, one had two ovulations, and another four. The time of ovulation could not be definitely ascertained, but in the cases of two ewes ovulation had occurred before the spaying operations, which were made on the fourth day following the injection of pregnant-mare serum. None of the eight ewes receiving pregnant-mare serum alone showed the behavior of estrus.

Two of the four ewes that received pregnant-mare serum and Progynon-B, simultaneously, ovulated. Both cases were single ovu-

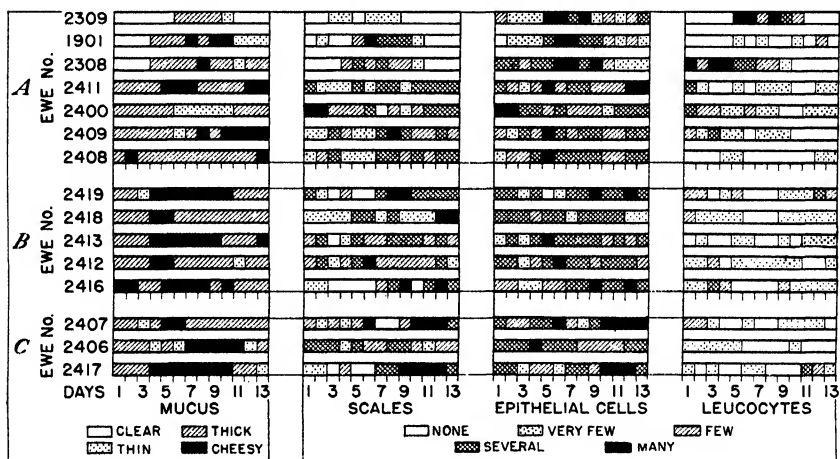


FIGURE 1.—Vaginal smear records of 15 injected ewes: A, 7 ewes given injections of 100 rat units of pregnant-mare serum on the second day, followed by ovulation; B, 5 ewes given injections of Progynon-B on the second day, estrus produced; C, 3 ewes given injections of Progynon-B on the second day, no estrus produced.

lations and ovulation was not accompanied by heat in either ewe. One of the two ewes not ovulating showed signs of heat.

Three of the four ewes that received pregnant-mare serum followed by Progynon-B ovulated. One of these came into heat 5 days after the injection of pregnant-mare serum (the time relation between heat and ovulation is not known). She was given 500 rat units of Progynon-B on the first and second day and 1,000 rat units on the third day following the injection of pregnant-mare serum. The fourth ewe neither ovulated nor came into heat.

Five of the eight ewes receiving Progynon-B alone came into heat. The length of the estrual period was impossible to determine in some cases as the ewes were spayed while still in heat. In the cases of three ewes which were spayed after going out of heat, two had estrual periods of 1 day and another of 2 days in length.

All four of the ewes receiving sheep anterior pituitary extract ovulated. The number of ovulations per ewe ranged from 2 to 13. None of the ewes showed any signs of estrus. Ovulation occurred in the ewe receiving pregnant-mare serum and sheep anterior pituitary

extract and in the one receiving pregnant-mare serum, sheep anterior pituitary extract, and Progynon-B, but neither ewe came into heat. No evidences of heat were seen in any of the ewes at any time other than following some endocrine treatment.

Vaginal smear records from 15 of the ewes are presented in Figure 1. Seven of these ewes ovulated following injections of pregnant-mare serum. The other eight were given Progynon-B. Five of these came into heat, while three showed no signs of estrus. Considerable variation was shown among the ewes, but the same general trends are indicated in the number of the different cell types, as well as in the consistency of the mucus in the three groups of ewes following the injections.

The most consistent changes are seen in the character of the mucus. These changes are most striking in the Progynon-B-treated ewes. The smears of these ewes became "cheesy" on the second to third day after the injection. The smear of the ovulating ewes tended to follow the same trend, as indicated by an increase in thickness after injections of pregnant-mare serum.

Changes in number of "scales" and epithelial cells appearing in the smears of the ewes following injections again show much individual variation within treatment groups and little, if any, difference between groups. Trends again are indicated and an increased number of both cell types were commonly found following the injections in all three groups. Leucocytes did not appear with sufficient regularity in the smears of the three groups to describe any general trend in their number following injections of either pregnant-mare serum or Progynon-B.

## DISCUSSION

Pregnant-mare serum, given in single subcutaneous injections of 100 rat units, produced ovulation rather uniformly in the anestrous ewes, but there were no signs of heat accompanying ovulation. It would, therefore, be necessary to force-mate or artificially inseminate such ewes at about the time of expected ovulation in order to have fertilization. (This is assuming that the ova are potentially fertile.) The situation is somewhat analogous to the "quiet" ovulations reported by Roux (9), who found ovulation without estrus occurring in Merino ewes during the anestrous period and particularly at the beginning and end of the breeding season.

The smallness of the dosage apparently does not explain the failure of these ewes to come into heat. Cole and Miller (2), (3) and McKenzie and Terrill (6) have given pregnant-mare serum at a level several times as high as that necessary to produce ovulation without causing the ewes to come into heat. Cole and Miller (3), however, have found that doses of 100 rat units spaced at 17-day intervals will produce both heat and ovulation. The latter procedure was not tried in this study.

The results from injections of anterior pituitary extract are similar to those obtained from the use of the pregnant-mare serum. All of the pituitary-treated ewes ovulated without a sign of estrus. Total doses given were 2.5 gm. (100 rat units) of pregnant-mare serum in single subcutaneous injections and 2.5 gram-equivalents of anterior pituitary powder in four daily subcutaneous injections of 0.5 gram-equivalent followed by an intravenous injection of the same amount.

Assay comparisons of sheep pituitary extract and of pregnant-mare serum may not be warranted because of the different nature of the assay curves for the two preparations. The ovarian weights, however, for the rats receiving 25 mg. of pregnant-mare serum were approximately equal to those for rats receiving 50 mg.-equivalents of anterior pituitary, and if this be accepted for comparison, the sheep treated with pregnant-mare serum received approximately twice as many rat units of hormone as those receiving pituitary. In spite of the greater potency of the pregnant-mare serum indicated by these assays, pituitary extract administered in total doses similar to the total doses of the serum gave a much higher ovulation rate. It is possible that dividing the total dose of pituitary extract into four subcutaneous and one intravenous injection may have been responsible for the increased ovulation rate. It is also possible that the ewe gives a greater response, per rat unit, to pituitary extract than to pregnant-mare serum. As the two materials were not injected in a similar manner, the correctness of these assumptions cannot be checked. Similar results have been obtained by Casida (1a), however, when the two hormone preparations were injected alike into cattle.

If a small amount of estrogen is responsible for the failure of the ovulating ewe to come into heat, it would seem reasonable to expect that supplemental estrogenic injections would be of value. However, as has been previously reported by Cole and Miller (3), combinations of both pregnant-mare serum and estrogens are usually not successful in producing both heat and ovulation. Although the number of ewes given this type of treatment in the present study is small, the results also indicate the difficulty in producing both heat and ovulation with pregnant-mare serum and Progynon-B.

The production of heat in the anestrous ewes by the use of Progynon-B was not uniformly successful in this study. The number of ewes treated was small, and there is little to indicate whether the higher or lower doses were more successful in producing estrus. These results are not surprising in view of the results of McKenzie and Terrill (6), who reported producing estrus in only 44 percent of 52 ewes given single injections of 800 rat units of Progynon-B.

The changes in the vaginal smear during artificially induced estrus or ovulation are in general similar to those described at natural estrus. No marked difference was shown between the smears of ewes which ovulated without showing signs of estrus and those of ewes which came into heat but did not ovulate. It would be impossible with the technique used in this study to determine by means of the vaginal smear whether ewes showing signs of heat had ovulated or not. It would appear that the changes in the vaginal smear are produced primarily by estrogenic substances and influenced little, if at all, by the hormone of the corpus luteum.

#### SUMMARY AND CONCLUSIONS

Thirty ewes were included in this study. Injections of pregnant-mare serum, sheep anterior pituitary extract, and Progynon-B, singly or in combination, were given to all of these ewes. Occurrence of heat was checked by the use of "teaser" rams and the ovarian stimulation was noted at the time of spaying.

Ovulation without heat was produced regularly by the use of either pregnant-mare serum or anterior pituitary extract. The number of ovulations per ewe was near normal in the ewes given pregnant-mare serum, but ranged from 2 to 13 in ewes given sheep anterior pituitary powder.

Progynon-B produced heat in five out of eight treated ewes.

Combinations of pregnant-mare serum and Progynon-B were generally unsuccessful in producing both heat and ovulation.

Vaginal smear records indicated no difference between the smears of ewes in which ovulation was induced without heat and the smears of ewes in which estrus was induced without ovulation.

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# THE CHEMICAL COMPOSITION OF FOREST FRUITS AND NUTS FROM PENNSYLVANIA<sup>1</sup>

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## INTRODUCTION

In the interest of wildlife conservation it is important to know the nutritive values of the food products of the forest, especially those which are available for the building up of nutritive reserves during the fall, to carry the wildlife over the critical winter period. Only after the various mast and browse foods have been evaluated is it possible, by proper forest management, to encourage the growth of the more desirable species. These are not necessarily the most nutritious ones, however, but rather are those which combine availability, productivity, nutritive value, and a capacity to resist decay. Upon this knowledge, and that of the food habits of the animals of the forest, wildlife management must to a large extent depend.

This paper seeks to represent, by means of chemical analysis, the nutritive value of 35 mast products from central Pennsylvania.

In making this study it was recognized that the possibilities of representing food values by chemical analysis are limited, and that the most significant information on the subject can be had only as a result of the use of the food products by animals. The mast foods are so diverse in character, however, some being concentrated foods capable of furnishing large parts of animals' diets, while many more contain little nutriment, and normally serve only as minor components of highly complex diets, that the only practicable method of conducting a general survey of the subject was by chemical analysis.

## METHODS

Beginning with the conventional feed analysis in terms of moisture, total nitrogen, ether extract, crude fiber, ash, and nitrogen-free extract, additional determinations of tannin, cellulose, lignin, available nitrogen, calcium, magnesium, and phosphorus were made.<sup>2</sup> Record was made (table 1) of the date and the approximate location of collection when this latter information was available. Three of the samples, namely, the nuts of the Italian chestnut, shellbark hickory, and hazel, were purchased in the market. The Italian chestnut was included with the idea that the analysis of this product might serve approximately to represent the American chestnut if and when it shall again attain a significant prominence as a mast food.

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<sup>2</sup> The materials analyzed were collected by L. J. Bennett, P. F. English, T. Kuhn, and R. McCain.

TABLE 1.—Name, date of collection, source, and portion analyzed of the fruit and nut products examined

## FRUITS

Common name <sup>1</sup>	Scientific name <sup>1</sup>	Date collected	Source in Pennsylvania	Portion analyzed
Apple, crab .....	<i>Malus coronaria</i> .....	Sept. 29, 1938	Huntingdon County	Whole fruit.
Bittersweet .....	<i>Solanum dulcamara</i> .....	Oct. 13, 1938	Centre County	Fruit, without calix.
Blackberry .....	<i>Rubus occidentalis</i> .....	Aug. 2, 1939	do .....	Whole berries.
Blackberry, Bailey's .....	<i>Rubus baileyanus</i> .....	Aug. 1, 1939	do .....	Do.
Blackhaw .....	<i>Viburnum prunifolium</i> .....	Sept. 29, 1938	do .....	Whole berries, unripe.
Blueberry .....	<i>Vaccinium</i> sp. ....	Aug. 1, 1939	do .....	Whole berries.
Cherry, wild .....	<i>Prunus virginiana</i> .....	Sept. 29, 1938	do .....	Whole fruit (1) and seeds (2).
Chokeberry, black .....	<i>Aronia melanocarpa</i> .....	July 31, 1939	do .....	Whole berries.
Chokeberry, red .....	<i>Aronia arbutifolia</i> .....	Oct. 18, 1939	do .....	Do.
Cucumber-tree .....	<i>Magnolia acuminata</i> .....	Sept. 23, 1938	Warren County	Whole fruit, three-fourths ripe.
Deerberry .....	<i>Accinium stamineum</i> .....	Aug. 29, 1939	Centre County	Whole fruit, unripe.
Dogwood, panicle .....	<i>Cornus femina</i> .....	Oct. 13, 1938	do .....	Whole berries.
Dogwood, red-osier .....	<i>Cornus stolonifera</i> .....	Sept. 25, 1939	do .....	Whole berries, half ripe.
Elder, American .....	<i>Sambucus canadensis</i> .....	Sept. 26, 1939	do .....	Whole berries.
Grape, frost .....	<i>Vitis cordifolia</i> .....	Oct. 13, 1938	Huntingdon County	Whole fruit.
Hackberry .....	<i>Celtis occidentalis</i> .....	Nov. 2, 1939	Centre County	Whole berries.
Hawthorn, cockspur .....	<i>Crataegus crus-galli</i> .....	Oct. 13, 1938	do .....	Whole fruit.
Juneberry .....	<i>Amelanchier canadensis</i> .....	July 7, 1939	do .....	Whole berries.
Mountain-ash, American .....	<i>Sorbus americana</i> .....	Sept. 29, 1938	Huntingdon County	Do.
Mountain-holly .....	<i>Nemopanthes mucronata</i> .....	Aug. 10, 1939	Centre County	Do.
Nannyberry .....	<i>Viburnum lentago</i> .....	Sept. 25, 1939	do .....	Whole berries, nearly ripe.
Spicebush .....	<i>Benzoin aestivale</i> .....	Sept. 29, 1938	Huntingdon County	Fleshy part (1) and seeds (2).
Sumac, smooth upland .....	<i>Rhus glabra</i> .....	Sept. 29, 1938	Centre County	Whole berries.
Sumac, staghorn .....	<i>Rhus hirta</i> .....	Oct. 18, 1939	do .....	Do.
Winterberry, Virginia .....	<i>Ilex verticillata</i> .....	Oct. 17, 1939	do .....	Do.

## NUTS

Buckeye, fetid .....	<i>Aesculus glabra</i> .....	Sept. 25, 1939	Centre County	Kernels.
Chestnut, Italian .....	<i>Castanea vulgaris</i> .....		Open market	Do.
Hazelnut .....	<i>Corylus americana</i> .....	Sept. 3, 1938	Centre County	Do.
Hickory, shell-bark .....	<i>Ilicoria ovata</i> .....		Open market	Do.
Oak, red .....	<i>Quercus rubra</i> .....	Oct. 20, 1938	Warren County	Kernels, with integument.
Oak, rock chestnut .....	<i>Quercus prinus</i> .....	Oct. 7, 1938	Huntingdon County	Do.
Oak, scrub .....	<i>Quercus ilicifolia</i> .....	Sept. 29, 1938	do	Do.
Oak, scrub chestnut .....	<i>Quercus prinoides</i> .....	Sept. 25, 1939	Centre County	Do.
Oak, white .....	<i>Quercus alba</i> .....	Oct. 13, 1938	do	Do.
Walnut, black .....	<i>Juglans nigra</i> .....		Open market	Kernels.

<sup>1</sup> Authority: BRITTON, NATHANIEL LORD, and BROWN, ADDISON. AN ILLUSTRATED FLORA OF THE NORTH-EASTERN UNITED STATES, CANADA AND THE BRITISH POSSESSIONS, FROM NEWFOUNDLAND TO THE PARALLEL OF THE SOUTHERN BOUNDARY OF VIRGINIA AND FROM THE ATLANTIC OCEAN WESTWARD TO THE 102D MERIDIAN. Ed. 2, rev. and enl., 3 v., illus. 1936. [N. Y. Bot. Gard.]

As the materials were received they were either immediately prepared for analysis or were stored for a limited time in sealed containers in refrigerating rooms. The fruits and berries were stored at approximately 40° F. and the nuts at temperatures below freezing.

In preparation for preliminary drying the fleshy fruits were sliced, and the nut kernels were cut into small pieces. The former were then dried in an air oven at approximately 50° C., and the latter in a vacuum oven at room temperature with reduced air pressure.

In the case of the wild cherry, separate analyses were made of the whole berries and of the seeds; and in the case of spicebush berries separate analyses were made of the outer fleshy integument and the seeds. The figures for the pulp and skin of the wild cherry, and for the whole spicebush berry, were calculated from the parts analyzed.

The oven-dry materials were transferred to a screened cabinet in which they were allowed to come to equilibrium with the moisture of the air. After 7 to 10 days they were weighed, and the loss of moisture from the fresh to the air-dry state was recorded.

The air-dry samples were ground in a meat chopper, and then extracted for 48 hours, the oily products (nuts, spicebush berry, etc.) with ether, and the sugary substances (blueberry, blackberry, etc.) with 95-percent ethyl alcohol. The air-dry residues were then ground in a micro Wiley mill, to pass a 20-mesh sieve, after which they were recombined, quantitatively, with their respective extracts.

The recombined materials were again dried in the air oven to remove the solvent, and were then rubbed through a 20-mesh sieve. Finally they were placed in the screened cabinet, and allowed once more to come into equilibrium with the moisture of the air; and after 7 to 10 days were bottled and sealed in readiness for analysis.

The methods of the Association of Official Agricultural Chemists (1)<sup>3</sup> were followed in the determination of moisture, total nitrogen, ether extract, crude fiber, total ash, nitrogen-free extract, tannin, calcium, magnesium, and phosphorus.

In determining moisture the samples were dried for 21 days in vacuum desiccators, without heat, in the presence of sulfuric acid. The samples thus dried were used in the determination of ether extract.

In employing the Kjeldahl-Cunning-Arnold method for total nitrogen, 4-percent boric acid was used as the receiving liquid, as proposed by Winkler (7).

Available protein was determined by the method of Horwitt, Cowgill, and Mendel (4), but this procedure was found not to be especially well adapted to the routine analysis of mast products because of excessive frothing during digestion.

Lignin was determined by the method of Ross and Hill (6) as modified first by Crampton and Maynard (2), and later by Crampton<sup>4</sup> by the use of Whatman, No. 50, acid-hardened filter paper in place of bolting cloth, and of preignited Celite Analytical Filter-Aid in place of the granulating agent consisting of chloroform and acetic acid. Cellulose was determined by the method of Kirschner and Hanak (5) as applied by Crampton and Maynard (2) to feeding stuffs.

## ANALYTICAL RESULTS

The results of the chemical analyses of the mast products are given in table 2 expressed on the dry basis, and in table 3 on the fresh basis. Comments will be made on the former alone, since, from the nutritive point of view, the moisture contained in the materials is simply a variable diluent.

<sup>3</sup> Italic numbers in parentheses refer to Literature Cited, p. 635.

<sup>4</sup> Unpublished data.

The sum of the values for lignin and cellulose was invariably higher (124 to 543 percent) than the single figure for crude fiber. This may be due to a loss of cellulose during the acid and alkali digestions involved in the determination of crude fiber; or the values for lignin and cellulose may conceivably be improperly high as a result of undetermined impurities.

The content of available protein was less than the crude protein in all products, indicating the presence of some fat-soluble, nitrate, amide or ammonia nitrogen. The error of regarding nonprotein nitrogen as protein nitrogen is of less significance when the foods are used by ruminants than when they are used by nonruminants, because the former have the capacity to utilize nitrogen from sources other than protein—presumably by virtue of the synthetic capacities of the bacteria of the rumen.

TABLE 2.—Percentage analyses (dry basis) of the fruit and nut products examined

FRUITS, DRY BASIS												
Common name	Crude protein	Ether extract	Crude fiber	Total ash	N-free extract	Available protein	Lignin	Cellulose	Tannin	Calcium	Magnesium	Phosphorus
Apple, narrowleafed crab	5.56	6.19	16.95	3.16	68.14	4.00	11.89	15.48	4.71	0.02	0.09	0.17
Bittersweet	15.19	28.77	8.20	3.44	44.34	11.88	8.98	10.22	1.35	.27	.29	.36
Blackberry	8.19	7.58	21.43	3.12	59.68	6.63	31.73	13.52	1.72	.15	.14	.21
Blackberry, Bailey's	6.75	6.08	24.14	4.31	58.72	5.56	28.58	15.49	2.04	.12	.17	.12
Blackhaw	4.13	11.93	10.28	2.58	71.08	3.09	24.68	7.70	5.94	.05	.08	.13
Blueberry	4.19	3.80	9.67	1.44	80.90	2.75	13.85	7.97	1.28	.04	.07	.07
Cherry, wild, whole	6.75	6.26	20.85	2.84	63.30	5.75	18.65	12.92	.60	.16	.07	.16
Cherry, wild, seeds	13.13	15.70	50.85	1.57	18.75	11.25	38.29	25.28	...	.18	.08	.19
Cherry, wild, pulp and skin	5.13	3.89	13.31	3.15	74.52	4.31	13.70	9.81	.75	.16	.07	.16
Chokeberry, black	5.00	3.44	12.56	2.71	76.29	4.19	39.80	9.80	3.78	.25	.12	.13
Chokeberry, red	5.25	3.80	9.24	2.52	79.19	4.38	36.13	8.48	7.31	.22	.21	.13
Cucumber tree	7.50	21.99	28.39	4.90	37.22	6.19	16.56	19.84	2.60	.23	.14	.20
Deerberry	3.75	5.45	11.17	1.62	78.01	2.50	11.46	8.57	1.71	.05	.05	.05
Dogwood, panicle	6.88	26.73	25.76	3.36	37.27	5.88	20.62	11.21	1.46	.21	.09	.15
Dogwood, red oster	6.94	12.02	26.42	3.40	51.22	5.56	27.12	12.01	1.58	.27	.19	.22
Elder, American	11.06	12.94	17.93	5.52	52.55	8.38	15.36	10.54	2.71	.13	.21	.36
Grape, frost	5.38	.87	13.43	2.75	77.57	4.19	14.50	7.45	1.99	.06	.08	.15
Hackberry	8.25	4.37	7.09	27.35	52.94	7.13	8.04	5.89	.82	12.42	.49	.22
Hawthorn, cockspur	2.81	3.29	32.83	3.69	57.38	2.56	20.44	23.32	3.39	.42	.10	.04
Juneberry	8.06	4.45	12.27	3.70	71.52	6.50	16.03	14.93	.41	.34	.21	.19
Mountain-ash, American	5.44	4.66	8.02	3.10	78.78	4.25	9.57	6.87	4.08	.10	.13	.16
Mountain holly	7.06	7.71	18.45	2.23	64.55	5.81	17.27	14.49	.98	.13	.13	.12
Nannyberry	4.13	8.88	7.18	2.06	77.75	3.63	32.32	6.60	1.57	.12	.04	.14
Spicebush, whole	11.94	50.73	5.23	5.74	26.36	9.94	4.04	5.98	1.35	...	.13	.30
Spicebush, seeds	18.19	56.21	7.45	2.20	15.95	17.06	5.21	4.91	.58	...	.16	.38
Spicebush, pulp and skin	8.56	47.82	4.05	7.62	31.95	6.13	3.41	6.54	1.75	...	.11	.26
Sumac, smooth upland	4.13	11.23	34.90	2.45	47.29	3.56	22.56	27.99	6.89	.16	.07	.16
Sumac, staghorn	5.44	14.54	30.31	3.01	46.70	4.88	21.66	27.50	4.41	.30	.15	.25
Winterberry, Virginia	6.13	5.10	15.57	2.62	70.58	5.31	9.49	12.61	.68	.13	.18	.10

#### NUTS, DRY BASIS

Buckeye, fetid	12.63	6.13	2.48	4.81	73.95	11.44	1.42	3.21		0.11	0.16	0.52
Chestnut, Italian	6.88	3.34	2.42	3.05	84.31	5.99	.38	3.69	0.19		.07	.15
Hazelnut	26.50	61.40	2.16	2.76	7.18	23.88	1.22	3.91		.29	.17	.40
Hickory, shell bark	13.31	74.36	1.51	2.01	8.81	12.13	7.74	2.63	.48	( <sup>1</sup> )	.16	.37
Oak, red	6.56	20.81	3.10	2.42	67.11	6.13	2.99	4.14	9.77	( <sup>1</sup> )	.07	.14
Oak, rock chestnut	6.94	5.05	2.62	2.22	83.17	6.25	2.50	3.53	10.43	( <sup>1</sup> )	.09	.15
Oak, scrub	10.25	19.99	3.00	2.12	64.64	9.56	4.00	3.76	11.28	( <sup>1</sup> )	.14	.19
Oak, scrub chestnut	7.63	6.30	2.42	1.98	81.67	6.88	6.56	3.19	4.43	( <sup>1</sup> )	.07	.18
Oak, white	6.25	6.32	2.47	2.64	82.32	6.00	2.64	3.24	5.58	( <sup>1</sup> )	.10	.16
Walnut, black	29.25	60.23	1.03	2.76	6.73	27.06	.87	2.01	.25	( <sup>1</sup> )	.27	.59

TABLE 3.—Percentage analysis of the fruit and nut products examined

## FRUITS, FRESH BASIS

	Moisture	Crude protein	Ether extract	Crude fiber	Total ash	N-free extract	Available protein	Lignin	Cellulose	Tannin	Calcium	Magnesium	Phosphorus
Apple, narrowleaf crab	87.0	0.75	0.80	2.20	0.41	8.84	3.50	1.55	2.01	0.61	0.01	0.01	0.02
Bittersweet	66.3	5.13	9.69	2.78	1.16	14.94	4.00	3.02	3.44	.45	.09	.10	.12
Blackberry	80.9	1.56	1.45	4.10	.60	11.39	1.25	6.06	2.58	.33	.03	.03	.04
Blackberry, Bailey's	79.6	1.38	1.24	4.93	.88	11.97	1.13	5.84	3.17	.42	.02	.04	.02
Blackhaw	57.1	1.75	5.12	4.41	1.11	30.51	1.56	10.59	3.31	2.55	.02	.03	.06
Blueberry	85.3	.63	.56	1.42	.21	11.88	.50	2.04	1.17	.19	.01	.01	.01
Cherry, wild, whole	64.5	2.38	2.22	7.41	1.01	22.48	2.06	6.63	4.59	.21	.06	.02	.06
Cherry, wild, seeds	11.5	11.56	13.90	45.02	1.39	16.63	9.94	33.90	22.38	-----	.16	.07	.17
Cherry, wild, pulp and skin	75.6	1.25	.95	3.25	.77	18.18	1.06	3.35	2.40	.18	.04	.02	.04
Chokeberry, black	75.6	1.25	.84	3.07	.66	18.58	1.00	9.72	2.39	.92	.06	.03	.03
Chokeberry, red	65.6	1.81	1.31	3.18	.87	27.23	1.50	12.43	2.92	2.51	.07	.07	.05
Cucumber tree	73.0	2.00	5.94	7.67	1.32	10.07	1.69	4.48	5.36	.70	.06	.04	.06
Deerberry	83.2	.63	.92	1.88	.27	13.10	.44	1.93	1.44	.29	.01	.01	.01
Dogwood, panicled	57.0	2.94	11.49	11.07	1.45	16.05	2.50	8.86	4.82	.63	.09	.14	.07
Dogwood, red-osier	68.4	2.19	3.79	8.34	1.07	16.21	1.75	8.56	3.79	.50	.09	.09	.07
Elder, American	76.4	2.63	3.06	4.24	1.30	12.37	2.00	3.63	2.49	.64	.03	.05	.08
Grape, frost	69.6	1.63	.26	4.08	.84	23.59	1.25	4.41	2.27	.61	.02	.03	.05
Hackberry	21.4	6.50	3.43	5.57	21.50	41.60	5.56	6.32	4.63	.64	9.76	.38	.17
Hawthorn, cockspur	62.0	1.06	1.25	12.48	1.40	21.81	1.00	7.77	8.86	1.29	.16	.04	.05
Juneberry	80.7	1.56	.86	2.37	.71	13.80	1.25	3.10	2.88	.08	.07	.04	.04
Mountain ash, American	73.7	1.44	1.23	2.11	.82	20.70	1.13	2.52	1.81	1.07	.03	.03	.04
Mountain-holly	59.2	2.88	3.15	7.53	.91	26.33	2.38	7.04	5.91	.39	.05	.05	.05
Nannyberry	53.8	1.94	4.11	3.32	.95	35.88	1.69	14.94	3.09	.73	.05	.02	.06
Spicebush, whole	62.3	4.50	19.14	1.97	2.17	9.92	3.75	1.52	2.26	.51	-----	.05	.11
Spicebush, seeds	28.4	13.00	40.26	5.33	1.58	11.43	12.25	3.74	3.51	.42	-----	.11	.27
Spicebush, pulp and skin	77.8	1.94	10.62	.90	1.69	7.05	1.38	.76	1.45	.39	-----	.03	.06
Sumac, smooth upland	8.1	3.81	10.32	32.07	2.25	43.45	3.25	20.73	25.72	6.33	.15	.06	.15
Sumac, staghorn	8.0	5.00	13.37	27.87	2.77	42.99	4.50	19.92	25.29	4.06	.28	.14	.23
Winterberry, Virginia	68.6	1.94	1.60	4.88	.82	22.16	1.69	2.98	3.96	.21	.04	.06	.03

## NUTS, FRESH BASIS

	Moisture	Crude protein	Ether extract	Crude fiber	Total ash	N-free extract	Available protein	Lignin	Cellulose	Tannin	Calcium	Magnesium	Phosphorus
Buckeye, fetid	52.7	6.00	2.90	1.17	2.27	34.96	5.44	0.67	1.52	-----	0.05	0.08	0.25
Chestnut, Italian	33.1	4.63	2.23	1.62	2.04	56.38	3.81	.25	2.47	0.13	-----	.05	.10
Hazelnut	2.6	25.81	59.80	2.10	2.69	7.00	23.25	1.19	3.81	-----	.28	.17	.39
Hickory, shellbark	2.2	13.00	72.72	1.48	1.97	8.63	11.88	.72	2.57	.47	( <sup>1</sup> )	.16	.36
Oak, red	38.2	4.06	12.87	1.92	1.50	41.45	3.81	1.85	2.56	6.04	( <sup>1</sup> )	.05	.08
Oak, rock chestnut	50.1	3.44	2.52	1.31	1.11	41.52	3.13	1.25	1.76	5.20	( <sup>1</sup> )	.04	.08
Oak, scrub	42.0	5.94	11.61	1.74	1.23	37.48	5.56	2.32	2.18	6.55	( <sup>1</sup> )	.08	.11
Oak, scrub chestnut	44.2	4.25	3.52	1.35	1.11	45.57	3.81	3.66	1.78	2.47	.04	.04	.09
Oak, white	47.3	3.31	3.33	1.30	1.39	43.37	3.13	1.39	1.71	2.94	( <sup>1</sup> )	.05	.09
Walnut, black	2.9	28.38	58.48	1.00	2.68	6.56	26.25	.84	1.95	.24	( <sup>1</sup> )	.26	.57

<sup>1</sup> Trace.

The values for nitrogen-free extract are of more indefinite significance than usual because they include the questionable tannin fraction, which reaches a maximum of 11.28 percent in the acorn of the scrub oak.

A lignin determination on the cellulose of the blackhaw revealed that a considerable portion of the lignin had not been removed during the acetic acid-nitric acid digestion. The percent of cellulose in the moisture-free sample was 7.70, and of this total approximately 16 percent appeared as lignin. Whether this means that the acid

digestion is incomplete, or that the lignin value is only an apparent one, remains to be determined.

#### FRUITS

Unless otherwise stated, the seeds of fleshy fruits were ground in with the remainder. This obviously gives consistent results for the products as used by animals which themselves grind the seeds, but yields improperly high results with animals which do not grind the seeds.

The fruits are predominantly carbohydrate foods; many are high in lignin and cellulose, and some are rich in protein and fat, mainly because of their seeds.

The bittersweet berry consists mainly of thin-walled seeds, with a thin covering of flesh of high moisture content. Its very high content of protein and ether extract, and relatively high content of mineral nutrients, constitute it a decidedly concentrated foodstuff.

The fruits of two species of blackberry, which are of much the same physical character as the bittersweet berry, appear to be of lower nutritive value. The composition, as stated here, represents the value of the blackberry to animals that grind the small, hard seeds and thus expose the kernels to digestion. The blackberry contains appreciable quantities of all the classes of nutrients determined, but is most notable for its high cellulose and lignin content.

The blueberry, deerberry, and juneberry are mainly carbohydrate foods. Aside from their content of nitrogen-free extract they are of moderately low value.

The value of the wild cherry depends largely on whether the shells are broken and the kernels utilized. The pulp and skin are relatively poor in protein and fat, but rich in nitrogen-free extract, while the seeds are moderately rich in protein, decidedly rich in fat, and poor in nitrogen-free extract. The pulp and skin are similar in composition to that of the blueberry, the deerberry, and the juneberry.

The berries of the mountain-ash and the two chokeberries contain nearly 80 percent of nitrogen-free extract, as well as approximately 5 percent each of crude protein and ether extract and therefore must be regarded as decidedly useful winter foods for animals to which they are acceptable. Both of the chokeberries contain high percentages of lignin.

The narrowleaf crab apple also is mainly a carbohydrate food, being relatively poor in protein, ether extract, and mineral nutrients, and rich in nitrogen-free extract. The high content of cellulose, lignin, and tannin are more suggestive of bark than of fruit. The product, however, appears to be characterized by a considerable value for purposes of maintenance.

In spite of its high crude-fiber content the fruit of the cucumbertree appears to be of high nutritive value to those animals that accept it. The ether extract is high, but the dense cellulosic network which lends rigidity to the fruit and its aromatic odor may render the fruit unacceptable to some animals.

The deerberry is a low-protein, low-fat, predominantly carbohydrate food, with no outstanding nutritive peculiarities.

The fruits of dogwoods, especially the panicked dogwood, are high in ether extract, and they have only a moderate content of protein and

mineral nutrients. The crude-fiber content is high, but not so high as to prevent these products from being important winter reserve foods.

The berry of the American elder has a composition that makes it nutritionally superior even to those of the dogwoods. Its lower crude fiber is associated not, as in many products, with a high content of nitrogen-free extract, but with a relatively high content of protein. The ether extract, although not as high as that of the panicle dogwood, is still higher than that of the red-osier dogwood.

The physical characteristics and composition of the frost grape place it in a class with the wild cherry pulp and skin, except that the ether extract is remarkably low. This means that it is mainly a carbohydrate food and therefore of value principally for maintenance purposes. As with other fruits containing a large proportion of seeds to pulp, the value of the frost grape depends largely on the method by which the animal disposes of the seed.

The hackberry is remarkable because of its unusual content of ash, and also of calcium, which, on the dry basis, amounts to 12.42 percent. Magnesium also occurs in an amount greater than in any other fruit or berry. The other constituents are present in moderate quantities, with the exception of lignin and cellulose, which are relatively low. Its magnesium content is twice as high as its phosphorus.

To those animals that can avail themselves of the whole fruit (flesh and seed) the blackhaw is moderately nutritious. It is most notable for its low protein and high lignin and tannin content.

The fruits of the mountain-holly, junberry, mountain-ash, nanny-berry, and winterberry are low-protein, low-fat products with the relatively high carbohydrate content usual among fruits.

The spicebush berry, whether whole or divided between pulp and skin, and seed, is unusual in that it contains approximately 50 percent of ether extract. This extract, however, is highly aromatic and may render the berry unacceptable to some animals. The content of protein is above average, while the percentages of crude fiber and nitrogen-free extract are decidedly low. This berry, therefore, possesses a decidedly high potential nutritive value.

The sumac berries also are rich in ether extract, though not nearly so rich as the berry of the spicebush. Their content of crude fiber is high, and the sum of the lignin and cellulose fractions amounts to approximately 50 percent. The nutritive value of these sumacs is presumably further lowered by their high content of tannin, but a positive qualification of sumac berries is the fact that they are unusually resistant to decay and therefore may serve as important food reserves, especially during the later winter months, when food is scarce.

That sumac berries are efficiently utilized, however, cannot be assumed with confidence, since Errington (3) found that not only sumac but also bittersweet berries, rose hips, dried wild grapes and sweet-clover seed, individually, failed to maintain captive bobwhites. At the same time it should be realized that maintenance on a single food is a severe test that many useful products cannot satisfy, and that a normally efficient diet might readily be made up of a variety of foods no one of which, by itself, is capable of maintaining the animal consuming it.



The cockspur hawthorn fruit is a decidedly inferior foodstuff. It is deficient in protein and fat, high in crude fiber, and relatively low in nitrogen-free extract. Furthermore, the nitrogen-free extract includes a considerable proportion of tannin.

#### NUTS

The analyses of all of the nuts represent the kernels only, the hulls being considered as without value.

Nuts in general are characterized by their concentration, as sources of nutriment, but there is much diversity in their content of protein, carbohydrate, and fat. The kernels are all low in lignin and cellulose.

Among the species analyzed, the black walnut, the hickory nut, and the hazelnut are in a class by themselves as high-protein, high-fat, and low-carbohydrate foods, with the hickory nut containing only about half as much protein as the walnut and the hazelnut, but decidedly more fat. The percentage composition of these three nuts is suggestive of that of animal flesh.

The chestnut and the acorns are relatively low in protein, crude fiber, and phosphorus, but contain substantial amounts of available carbohydrate. Three of the five acorns are also low in fat.

The nuts analyzed were all exceedingly low in calcium, the hazelnut being the highest in this constituent, and were also low in phosphorus; but among these species the buckeye, hazelnut, hickory nut, and black walnut were richer in this element than were the chestnut and the acorns. The buckeye was richest of all in ash.

The acorns were all high in tannin content, the red oak, rock chestnut oak, and scrub oak being the highest. The nutritive status of tannin, for wildlife, remains to be determined. Obviously foods which are rich in this constituent are acceptable to many kinds of animals, but not to others.

#### SUMMARY

Chemical analyses are presented of 35 mast foods, the conventional food analysis being supplemented by determinations of tannin, cellulose, lignin, available protein, calcium, magnesium, and phosphorus.

The sum of the lignin and cellulose fractions was invariably found to be higher than the value determined for crude fiber.

In all products the content of available protein was less than that of crude protein.

The values for nitrogen-free extract of mast foods are of more questionable significance than the corresponding values for foodstuffs in general because they include the tannin fraction, which reached a maximum of 11.28 percent, on the dry basis, in the acorn of the scrub oak.

The fruits and berries are, for the most part, relatively rich in nitrogen-free extract and much less rich in protein and ether extract. They are of only moderate nutritive value, and serve mainly for energy production. The nuts are of greater value, especially because of their content of protein and fat.

In contrast to most fleshy fruits, those of bittersweet, cucumber-tree, panicked dogwood, and spicebush are remarkably rich in ether extract, and are, therefore, relatively concentrated foods.

The hazelnut, hickory nut, and black walnut are very concentrated foods, containing much ether extract and protein, and very little nitrogen-free extract. Among nuts they are relatively rich in phosphorus. Almost all of the nuts analyzed were exceedingly low in calcium, and they usually contained much more magnesium than calcium.

Chestnuts, buckeyes, and most acorns are oil-poor and are relatively rich in nitrogen-free extract; they are not so rich in protein as are the oil-bearing nuts.

The products richest in tannin were the acorns, chokeberries, sunac berries, blackhaw, narrowleafed crab apple, and mountain-ash berries.

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## DISTRIBUTION BY THE SAP STREAM OF SPORES OF THREE FUNGI THAT INDUCE VASCULAR WILT DISEASES OF ELM<sup>1</sup>

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### INTRODUCTION

Several factors common to the two native vascular wilt diseases of the American elm (*Ulmus americana* L.), induced respectively by *Verticillium dahliae* Kleb. and *Dothiorella* (*Cephalosporium*) *ulmi* Verrall and May, and particularly conspicuous in the introduced Dutch elm disease, induced by *Ceratostomella ulmi* Buisman, suggest that in these diseases the wilt-inducing fungi are distributed within the tree chiefly by drifting spores rather than by mycelial growth in the vascular elements. Thus, the discontinuous pattern of the discoloration induced by these fungi in the infected vascular elements suggests distribution by randomly scattered spores rather than by an uninterrupted mycelial growth. Histological studies of the Dutch elm disease reported by Wollenweber (38, 39),<sup>3</sup> Buisman (9), Tubeuf (36), Banfield and Smith (4), and Clinton and McCormick (11) disclose a fragmentary, discontinuously distributed, sparse mycelium in the tracheae of infected trees. Conidia have been reported by Wollenweber (39) to be produced and randomly scattered in such vessels; conidia and yeastlike cells have been reported by Banfield and Smith (4).

The present investigation was designed to ascertain whether spores of the three fungi known to induce vascular wilt diseases of elm are distributed by the sap stream and, if they are so distributed, to show the extent of and the approximate time required for such distribution at various seasons of the year.

*Ceratostomella ulmi* was used mostly, because (1) it produces spores in enormous numbers in culture as a result of yeastlike multiplication; (2) it can be isolated from infected tissue with less difficulty, owing to the more rapid growth it makes from cultured tissue, than *Cephalosporium* or *Verticillium*; and (3) it is more readily isolated and identi-

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<sup>3</sup> Italic numbers in parentheses refer to Literature Cited, p. 679.

fied by virtue of the coremial fructifications to which it gives rise directly on wood or on cultured tissue.

Preliminary reports of this work have been presented before the American Phytopathological Society (2, 3, 4).

#### REVIEW OF LITERATURE

Wollenweber (39, pp. 2-3) in 1929 said of *Ceratostomella ulmi* in the elm:

Der Pilz lebt hauptsächlich in den der Wasserbewegung dienenden Gefäßen des Splintholzes. Er durchwächst dieselben jedoch nur spärlich mit seinen Fäden. Vielmehr geht er so leicht zur Fruktifikation über \* \* \* die bei hefeartiger Sprossung besonders schnell erfolgt, dass er sich mit seinen Konidien vom Saftstrom in der Pflanze passiv treiben und dadurch überallhin im Baume verschleppen lassen kann.

See also Wollenweber and Stapp, 1928 (41, pl. 2, figs. O and P).

May (22, p. 13) in 1934 reported spores of *Ceratostomella ulmi* to have been passed through "3 feet of elm branch in a few minutes," and from the speed with which the organism moved upward in his inoculations (8 inches per day) he too surmised that "such rapid extension can scarcely be due to simple mycelial growth, but \* \* \* is more likely due to the dispersal of free spores in the water-conducting vessels."

Tubeuf (36, p. 167) in 1935, though fascinated by such a singular mechanism, searched the literature in vain for proof, and said:

Ich habe vergeblich in der Literatur den Nachweis dieses einzigartigen Vorganges gesucht und kann mir die wasserarmen, verstopften und leitungsunfähig gewordenen Tracheen gar nicht zu der ihnen-zugeschriebenen Rolle geeignet denken.

On March 10, 1936, A. L. Smith<sup>4</sup> demonstrated the presence of spores of *Ceratostomella ulmi* in drops of water that dripped from the base of a 30-foot section of the bole of an American elm 7 inches in diameter breast high, to the upper end of which he had attached, 70 minutes earlier, a reservoir of spore suspension of this organism.

Buisman (10) in 1936 sucked spores of *Ceratostomella ulmi* through stems of various elm species with the Melhus "fluometer" by vacuum pressure and air pump. The maximum stem length through which she passed the spores was 88 cm., in *Ulmus elliptica*; the minimum length was 29 cm., in *U. foliaceae* No. 24, the resistant variety now called Christine Buisman. Two days after injection of spores with a hypodermic syringe she recovered the organism from stem tissue as much as 70 cm. above injection points on seedling trees of *U. americana*; but from branches that were injected in this manner immediately after excision from trees of this species, the maximum distance at which she recovered the fungus 2 days after injection was 15 cm. She concluded (10) that the water stream in the plant certainly plays a role in the spreading of the disease and assumed that the spores were carried along by the water stream in the plant.

Went (37) in 1937, using a similar injection-isolation technique, reported recovery of *Ceratostomella ulmi* 20 cm. below injection points on seedlings of *Ulmus americana* and *U. glabra*, and 30 cm. below injection points on branches of the less susceptible *U. pumila*, 2 days after injection. Records of the length of the stem axes injected are not reported by Buisman or Went.

<sup>4</sup> Unpublished records in the laboratory of the Division of Forest Pathology, Bureau of Plant Industry, U. S. Department of Agriculture, at Morristown, N. J.

Radulescu (29, p. 631), who stepped elm branches cut under water in spore suspension, said:

Nach 14 Stunden konnte durch Abimpfen auf Agar sowie durch mikroskopische Untersuchung festgestellt werden, dass die Konidien auf eine Strecke von 50 cm von der Schnittfläche in den Leitungsbahnen aufgestiegen waren.

The length of the branch is not given. He hung a similar branch upside down, connected the cut end to a reservoir of spore suspension by rubber tubing, then cut off the ends of small twigs and leaves. He reported that *Ceratostomella ulmi* was cultured from drops of water that emerged from certain cut twigs up to 55 cm. from the injection surface, and that 2 months later (29, p. 633) "waren im jüngsten Jahrring die für die Ulmenkrankheit kennzeichnenden braunen Steifen längs der Tracheen zu sehen."

Radulescu finally held the tips of excised and unexcised branches under a spore suspension. The 30 to 35 submerged leaves of the branch tip were (29, p. 634)—

bis etwa zur Hälfte abgeschnitten, wodurch die Leitungsbahnen der Blatternerven geöffnet wurden. \* \* \*

Nach 16 Stunden wurde die Untersuchung vorgenommen. Mikroskopisch wurden fast in allen Leitungsbahnen des letzten Jahrringes Konidien beobachtet. Ausserdem wurden an verschiedenen Stellen kleine Holzproben auf Agar abgeimpft.

Positive isolations are recorded throughout all parts of the diagram of this 2.8-m. branch, even to the base. Positive isolations were made only from the "juengsten Jahrringe der Triebe und Blattnerven angegeben."

Radulescu also cut under spore suspension leaves on the tips of the terminal branches of standing trees and held them under this suspension for 6 days. Isolation studies then made revealed the presence of the organism, according to diagrams presented, to the tips of small branches at the base of the two trees studied, respectively 4 and 8 m. in height.

In similar studies on oak and birch, Radulescu recovered the organism at 32 and 40 cm. respectively from leaf injection points 7 days after injection, and at 30 cm. from leaf injections on beech 17 days after injection.

#### MATERIAL AND METHODS

Spores of the three elm wilt-inducing fungi (*Ceratostomella ulmi*, *Dothiorella (Cephalosporium) ulmi*, and *Verticillium dahliae*) were injected or inoculated into the bases or tops of tall *Ulmus americana* and *U. fulva* Michx. trees at various seasons of the year. The distribution of these fungi was then traced from the presence of spores in sap samples taken from representative points throughout the injected trees or from the discoloration induced in the tracheae of the trees. Evidence concerning the probable speed of distribution was obtained by observation of the movement of highly colored suspensoids and stained yeast cells, which were injected into similar trees immediately after the removal of the bark.

Only trees in good vigor, which displayed no foliar symptoms of disease, were used. In view of the eradication program, special care was taken to see that none of the trees used experimentally became a hazard for the spread of the disease and all were promptly destroyed after use.

## INJECTION PROCEDURE

Large volumes of spore suspension, chiefly of *Ceratostomella ulmi*, were injected into the bases or tops of *Ulmus americana* and *U. fulva* trees 10 to 67 feet high, before, during, and after the leafy seasons of 1936 to 1939, inclusive. The spores were injected through chisel cuts made under the surface of aqueous suspensions held around the tree at the point of injection by means of cone-frustum pans (fig. 1). The

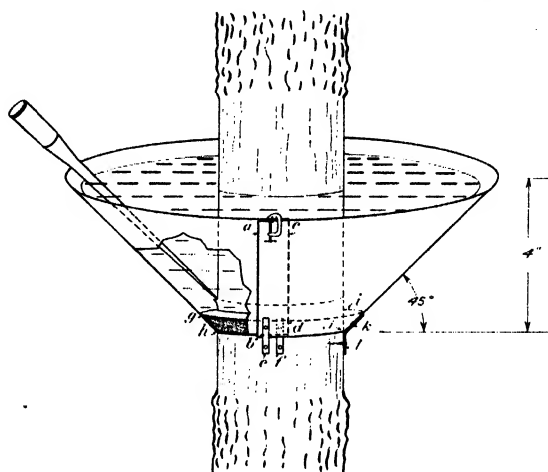


FIGURE 1.—Tree-injection pan (45° cone frustum 4 inches deep): *ab* and *cd*, Free ends of pan; *e* and *f*, nails fastening ends of pan to stem; *ghij*, layer of grafting wax (or plastic clay) sealing lower edge of pan; *kl*, metal strap fastening pan to stem.

chisel cuts were made to a depth of  $\frac{1}{4}$  to  $1\frac{1}{2}$  inches around the entire circumference of the tree at the point of injection. According to Huber (21), the vessels of the outermost ring are the most active in conduction. All of these vessels were injected by this procedure. The injection cuts were kept covered by spore suspension throughout the duration of the injection. The injection time varied from several minutes to several days. The suspensions used contained between 1 million and 5 million spores per cubic centimeter. One hundred cubic centimeters to 89 liters of suspension was taken up by individual trees in injection periods of 20 to 120 minutes.

The injection pans used to introduce large quantities of spore suspension into the trees were 45° cone frustrums, 4 to 6 inches deep, ranging from 3 to 20 inches in diameter at their smaller ends (fig. 1). Any stem within the diameter range covered could be fitted by the series of sizes, each being 1 inch larger than the one preceding it. Each pan was cut in one piece from a sheet of 28-gage sheet iron. At its lower end metal straps (*kl*) were riveted and soldered to the outer face of the pan. The pan was fastened by nailing these straps to the stem. The free ends of the pans (*ab*, *cd*) when pulled apart permitted the pan to be slipped around the stem of standing trees. One end (*cd*) was fastened at its lower edge to the stem by a nail at *f*; the other end overlapped and was pulled tight around the stem and was fastened

to the tree by a nail at *e*, to the inner face of the pan at *cd* by a strip of adhesive tape, and at the upper edge of the pan (*ac*) by a C-type screw clamp. This type of pan and injection procedure were used by Shevyrev (31). A less satisfactory type of pan, used in the early part of this work, was suspended by wires from nails and had a flexible rubberized cloth cemented to its base. The cloth was bound at its base to the stem by adhesive tape (fig. 2, *A* and *B*).

The pans were sealed to the tree and disinfected by the alcohol-flame procedure prior to each injection. The outer bark at the injection point was shaved smooth to facilitate sealing. The crevices usually present at the lower end of the pan, because of uneven curvature of the stem, were sealed with a layer of grafting wax or plastic clay (fig. 1, *ghij*). The latter was by far the more satisfactory. So sealed, the inner face of the pan and that portion of the stem from which the outer bark had been removed were flamed with alcohol. This provided adequate disinfection of surfaces with which the pure-culture spore suspension subsequently came in contact.

Spores suspended in a few drops of sterile water were injected through cuts made at the tops or bases of similar trees with a 1-inch chisel. The cuts were made deep enough to insure penetration of the outer ring of vessels. Drops of spore suspension were placed on the injecting chisel in such a manner as to come in contact with the severed vessels as the chisel was withdrawn. Additional drops of spore suspension were added until the cuts had absorbed one-half of a cubic centimeter of the suspension, or during the dormant season, until the chisel-cut crevices were filled.<sup>5</sup>

#### INOCULATION PROCEDURE

Similar trees were inoculated. Chisel cuts were made at one or several points in the trees to a depth which insured that the outer ring of conducting vessels would be severed. Five to ten minutes later the cut surfaces were brushed with a camel's-hair brush, which just previously had been moistened, but not saturated, with spore suspension. It was assumed that spores would not be introduced into the functional vessels by this procedure. Results indicated that none were. Spores can be distributed by the sap stream in trees so treated only after the hyphae that arise from the inoculum have penetrated into functional vessels and have there released new conidia (4).

#### TREATMENT OF TREES AFTER INOCULATION OR INJECTION

The experimental trees were felled 15 minutes to several months after spores had been introduced into them. Those felled within 3 weeks after inoculation or injection were immediately defoliated and sectioned if spore distribution was to be traced by the discoloration procedure. They were cut at roughly one-third, two-thirds, and five-sixths of their height. They were then left lying in the shade, usually on the ground where they fell, until the discoloration induced by the spores developed. Spore distribution in trees felled 21 or more days after injection or inoculation was usually traced immediately by the discoloration procedure, and the trees were then destroyed. The leaves were not removed from such trees.

<sup>5</sup> During the dormant season, water was drawn into such cuts so slowly that it was frequently impracticable to stand by until one-half of a cubic centimeter of suspension had been absorbed. During the leafy season, one-half of a cubic centimeter was usually absorbed within a few seconds from a quarter-inch chisel cut on elm.



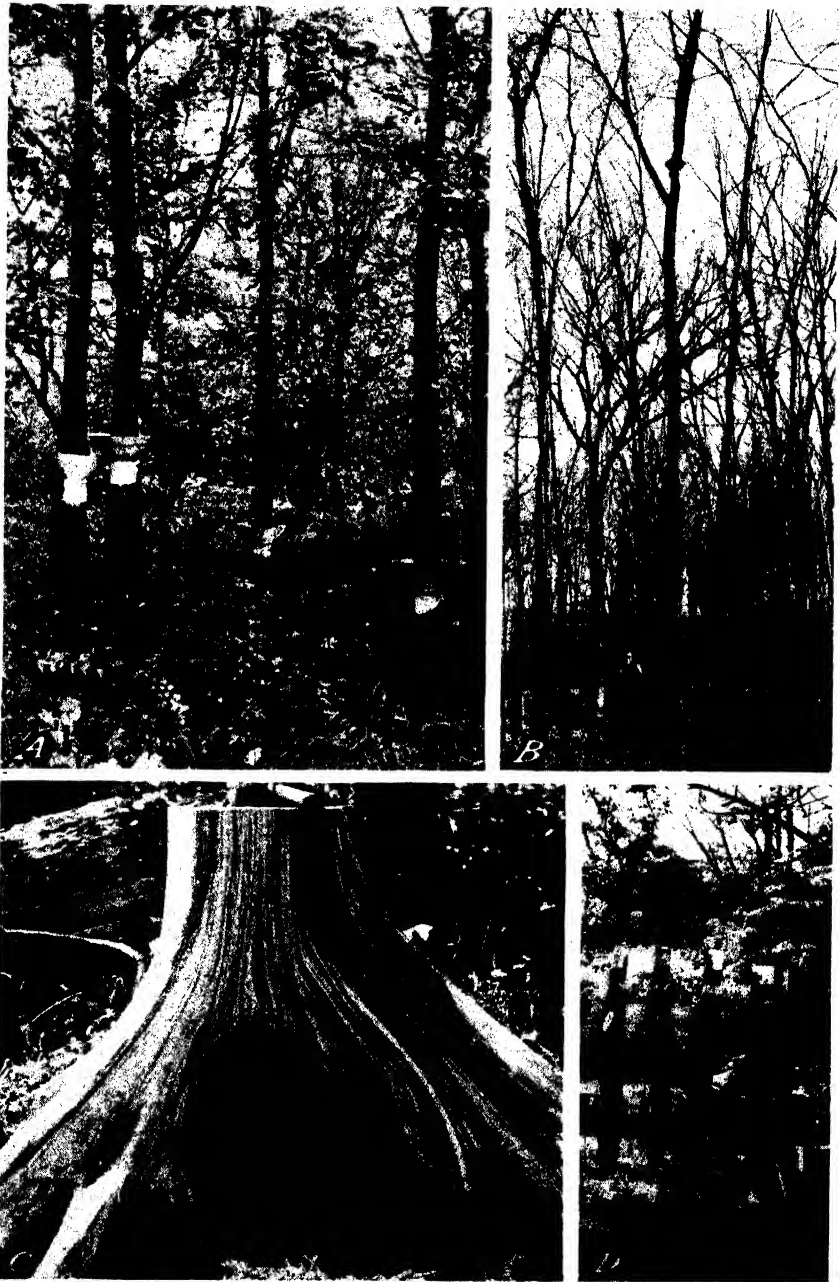


FIGURE 2.—A and B, Injection pans in place at the base and at the top of the boles of American elms; C, streaks of discoloration produced on the surface of the wood of a diseased elm by invasion of the new ring of vessels by *Ceratostomella ulmi*; D, sections of spore-injected trees in position for displacement of sap and spores by water held in watertight collars at top of sections.

When spore distribution was to be traced by the sap-displacement method, trunk and leader branches were cut into 3- or 4-foot lengths and taken directly to the laboratory. The small leafy branches and twigs were then lopped off with a knife or a hatchet. Before cutting, the stem surfaces and implements were alcohol-flamed. Immediately after cutting, the cut ends were painted with or immersed in a hot vaseline-paraffin mixture as protection against the introduction of contaminants.

#### METHODS OF TRACING DISTRIBUTION OF SPORES IN THE ELM

##### SAP-DISPLACEMENT PROCEDURE

The distance to which the spores were distributed in the tree was traced by the sap-displacement procedure from the presence of the injected spores in sap displaced from various parts of the tree.

Sap was displaced with water by gravity from each 3- or 4-foot section of the injected stem. The bark was shaved smooth at the upper end of each section; the wax was cut from the outer several rings of large vessels at this end of the sections; the surfaces were flamed; a sterile metal collar was bound with adhesive tape around the stem over the smoothed bark, and then sealed with grafting wax. The collar attached in this way formed a reservoir to which sterile water was added after the log had been suspended vertically (fig. 2, *D*). Before suspending the logs, their lower ends were pointed with an axe, then alcohol-flamed. Liquid dripped from the lower ends of these logs within a few minutes; it was caught in sterile flasks and later was examined for spores. It was assumed that the water would wash out many spores or would displace such sap and spores as might be in the stem section in those vessels through which spores had been distributed. Usually from 50 cc. to 500 cc. of liquid, depending on the diameter of stem, was collected in this manner from each stem section.

The sap and water displaced from the stem sections were then immediately centrifuged, examined for spores, and cultured. The living cells in the sap samples were concentrated in a 0.2-cc. volume of the liquid by centrifuging. Microscopic observation was made of drops of this concentrate for the spores injected. Spores of *Ceratomyella ulmi* were readily recognized if present in their yeastlike form (fig. 3). Ultimate identification of the spores was obtained from the character of the mycelial growth and fructifications to which they gave rise when isolated on acidified malt agar. To isolate them each concentrated sap sample was diluted with sterile water. Each of three to five Petri dishes containing sterile solidified agar was then flooded with 1 cc. of the diluted sap sample. Proper dilution was computed from the number of spores counted in the concentrated sap samples. Three dilutions were cultured in each case, i. e., the dilution considered optimum and that on either side thereof, in a series of dilutions each 10 times greater than the one preceding it. The plates were examined for colonies of the fungus injected after they had incubated a suitable time. The number of colonies of the injected fungus that appeared in the plates was an index of the number of viable spores of this fungus contained in the liquid displaced from the stem section in question. This in turn was demonstration that the spores injected had been distributed to the horizontal level of at least the lower end of this stem section.

## DISCOLORATION PROCEDURE

Several days to weeks after the injection of the spores of any of these fungi into *Ulmus americana* or *U. fulva*, either in the spring or in the leafy season, the vessels occupied by the injected spores showed conspicuous discoloration (fig. 2, C). The locus of distribution and the distance to which the spores had been distributed were then apparent from inspection of the vessels. Isolations were made from this discolored tissue at representative points in each tree, in order to confirm the association of the injected organism with the discoloration. This method also served as a further check when the sap-displacement method was employed, since not all the spores introduced by the injection were displaced with the sap.

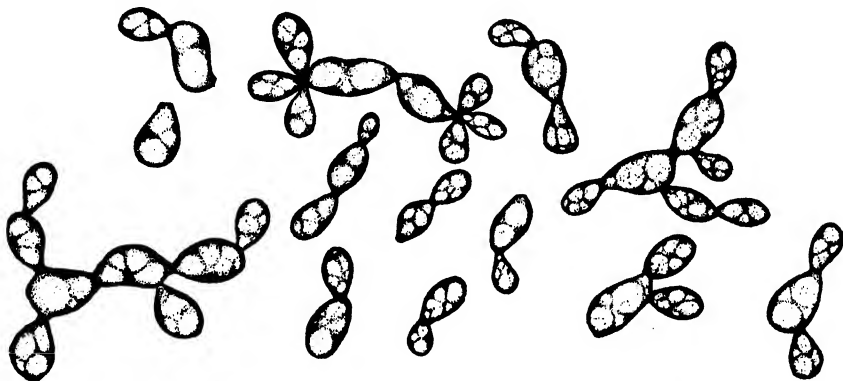


FIGURE 3.—Yeastlike cells found in sap displaced from the vessels of trees injected with a spore suspension of *Ceratostomella ulmi* and also in sap displaced from naturally diseased trees. These spores are typical also of those produced in liquid culture by *C. ulmi*.  $\times 1,550$ .

Discoloration develops, during the spring or during the leafy season, in those functional vessels of American elm to which any one of these organisms has gained access. No exceptions were found during this work. This discoloration is of a light reddish-brown to dark-brown color. It arises in and centers around such points in the functional vessels as become occupied by the fungus. The initial color may be of low intensity but can usually be detected within 3 days after such occupation. This initial color is usually reddish brown, sometimes a faint steel blue. It becomes more intense as time passes. The ultimate color generally is a conspicuous reddish to intense dark brown. The streaks formed (fig. 2, C) by such discolored vessels are a characteristic sign of the wilt diseases induced by *Ceratostomella ulmi*, *Dothiorella ulmi*, and *Verticillium dahliae*, respectively.

The extent of the distribution of the injected spores in the vessels was readily determined by inspection for discoloration several days to weeks after injection. Distribution was practically limited to the ring of large spring vessels produced during the season of injection. This ring lies essentially on the surface of the wood during the early weeks of the season, i. e., it is covered by only a thin layer of translucent, unignified new tissue. After the bark had been removed in the early part of the season, the vessels of this ring that were occupied

by the injected spores appeared as brown streaks, in striking contrast to a background of clear, whitish wood (fig. 2, *C*). Since the intensity of the discoloration induced in the vessels increased up to a certain point as time passed, determinations of spore distribution by this method were more readily and more accurately made 2 to 4 weeks after injection. Invasion of the tissue by organisms that penetrated through beetle galleries or drying bark areas after some time usually induced general tissue discoloration, which obscured the discoloration induced in the vessels by the injected fungus. Since this became progressively more extensive with time, 5 weeks during the leafy season was the maximum period feasible in these experiments for development of significant discoloration. After inspection for discoloration, stem samples were taken from representative points for cultural tests, and the trees were destroyed.

When observed in cross section the vessels occupied by the fungi appeared as small spots of discoloration in otherwise clear wood. (See fig. 7.) The color after a time diffused very slightly into contiguous tissue (fig. 4, *B*). From such discolored vessels the injected fungi were readily isolated. In general, with few exceptions, *Ceratostomella ulmi* cannot be isolated from nondiscolored vessels and tissues.

Isolation of the injected organism from the discolored tissue was accomplished by placing chips of this tissue on acidified potato-dextrose or malt agar. After 3 to 14 days' incubation at 24° C., any of these fungi could easily be identified by their characteristic conidial fructifications and growth. When isolation of *Ceratostomella ulmi* by this technique was difficult because of contaminants, the wood chips were held on moist filter paper incubated at 18° for 3 weeks, at the end of which time examination for coremia was made. Representative coremia were then streaked over the surface of sterile nutrient-agar plates. Colonies typical of *C. ulmi*, with characteristic "dog-ear" conidial formations, were the final criteria for identification of the coremia and of the presence of *C. ulmi* in the tissue cultured.

## DISTRIBUTION OF SPORES IN ELMS INJECTED STUMP HIGH

### SPORES OF CERATOSTOMELLA ULMI

#### TREES INJECTED IN SPRING BEFORE MATURATION OF NEW RING OF LARGE VESSELS

Spores of *Ceratostomella ulmi*, when injected at stump height into native elm trees during the spring flowering period or dormant season, were distributed only a few inches above the points of injection. None were recovered by the sap-displacement method at any distance above the injection points. Sap samples were examined and cultured from all parts of the bole of these trees. Discoloration was induced around certain small vessels of the wood (fig. 4, *D*) of all growth sheaths penetrated by the injection chisel in all trees injected in this manner during the spring and leafy season. Color in this system of vessels did not extend beyond 18 inches above the injection point in any elms at any season in those injection experiments in which the trees were felled within 1 day after injection. *C. ulmi* was recovered

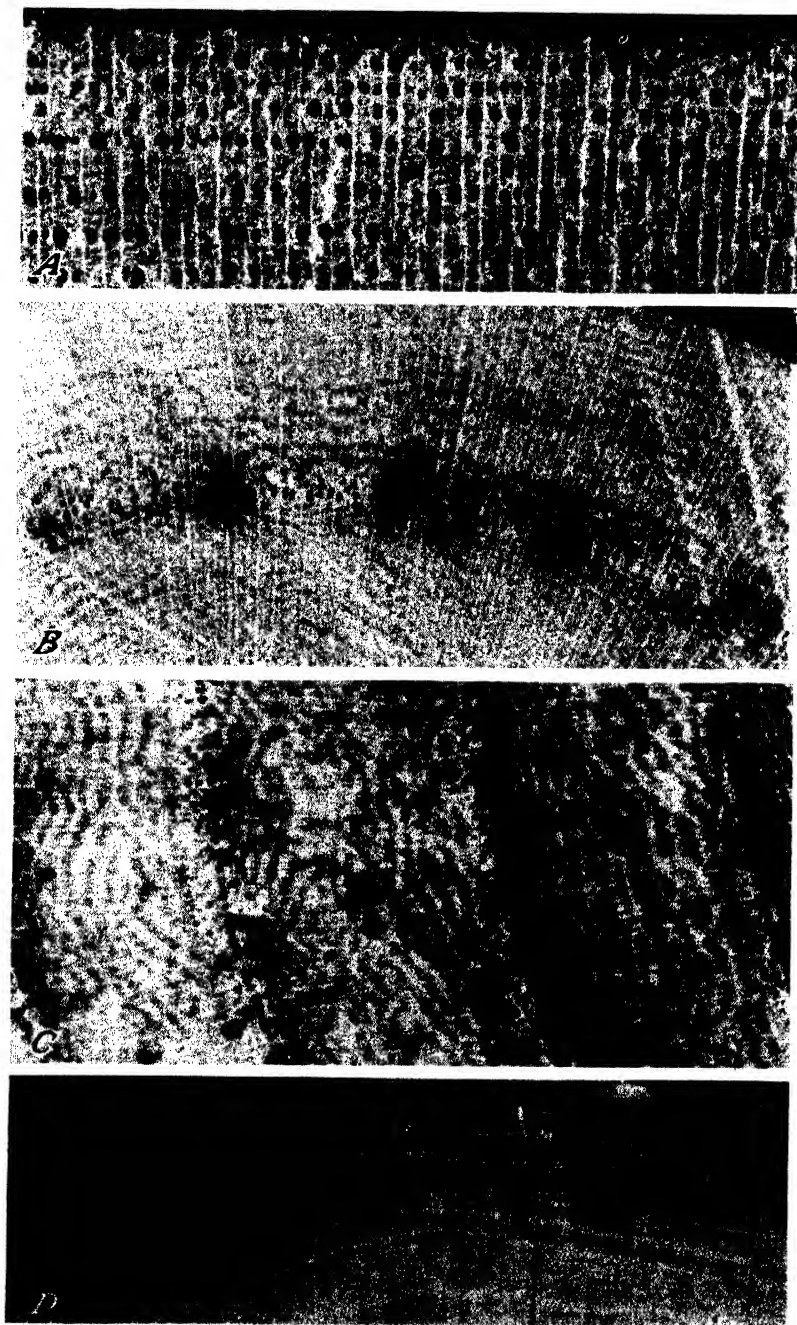


FIGURE 4.—For explanatory legend see opposite page.

from these discolored areas in early trials at only a few inches above the injection point (table 1). It was recovered in later trials up to the limits of the discoloration induced by the spores injected.

#### TREES INJECTED IN EARLY SUMMER OR MIDSUMMER

##### SMALL TREES

Spores of *Ceratostomella ulmi* were injected a few inches above the ground, that is, at stump height, into the main stem of small native elms during the early half of the leafy season. These trees ranged up to 20 feet in height. The injections were made by the usual pan and chisel-cut procedure and ranged from 20 to 60 minutes in duration. The trees were felled and defoliated immediately after injection. Discoloration induced by the spores extended from the injection point up the main stem, and out to within a few feet of the ends of the major branches of the crown (fig. 5, C). *C. ulmi* was recovered at representative points up to the limits of discoloration in all the trees that were cultured (table 2). No attempts were made in these experiments to recover the spores from these trees by the sap-displacement method.

##### TALL TREES

Spores were recovered by the sap-displacement method from the tops of tall elm trees 3 hours after injection during trials conducted in June 1936 (table 1). Three trees, 6 to 7 inches in diameter breast high, were injected 6 inches above the soil line between 10:30 a. m. and 1:30 p. m., June 15, 1936. The weather was ideal for active transpiration in northern New Jersey (temperature, 75° F.; relative humidity, 45 percent; wind, slight). Four and one-half gallons of

#### EXPLANATORY LEGEND FOR FIGURE 4

A, Transverse section of the bole of tree 116, taken 5 feet above the point of injection with sterile water. Tyloses occlude all inner rings of large vessels. The vessels of the outer ring are clear and show no discoloration. Injected August 3, 1937; photographed February 10, 1938. × 10.

B, Spots of typical discoloration induced by *Ceratostomella ulmi* in the new vessel ring as seen in a representative transverse section of stem (branch G, tree 107) taken at 56 feet above the point of spore injection. Each spot involves one to several vessels. *C. ulmi* was isolated from such spots in nearly all trials, but not from nondiscolored areas. Tyloses have formed subsequent to the injection and occlude practically all vessels of the ring. Injected August 9, 1937; photographed February 10, 1938. × 5.

C, Transverse section of stem taken 35 feet above the point of injection with acid fuchsin from a leafy elm chemically girdled February 1937. The relatively nondiffusible dye remained confined to vessels active in sap flow, i. e., certain small tracheae of the wood in many outer rings. Photographed 1 hour after injection July 16, 1937. × 4.

D, Spots of discoloration induced by *Ceratostomella ulmi* in the bole of an elm injected with spore suspension. Transverse section taken 6 inches above the point of injection. The spots center around certain vessels of the wood in all growth sheaths penetrated by the injection cut. No discoloration developed in the spring ring vessels severed by the injection cut. Vessels of the new ring had not matured. *C. ulmi* was consistently isolated from the discolored areas; it was not isolated elsewhere. The discolored spots did not occur beyond 18 inches above or below the injection point. Injected May 7, 1937; photographed June 1, 1937. × 1.

the spore suspension was taken up by the three trees during the 3-hour injection period. Fifty to one hundred cubic centimeters of liquid was then displaced from the various sections of stem above the injection points (table 1) for trees 8 and 9. Each sample was

TABLE 1.—Distribution of spores of *Ceratostomella ulmi* injected<sup>1</sup> stump high into American elms at various seasons, 1936

Tree No.	Injection date	Seasonal condition of trees	Time between injection and sectioning <sup>2</sup>	Tree dimensions	
				Diameter breast high	Height <sup>3</sup>
			Days	Inches	Feet
1.....	Apr. 1.....	Flowering.....	3	4	22
2.....	do.....	do.....	3	5	18
3.....	May 1.....	Leaf buds unfurling.....	3	5	20
4.....	do.....	do.....	3	5	20
5.....	Apr. 10.....	Flowering.....	28	3	18
6.....	May 28.....	Rapid shoot elongation.....	2	7	35
7.....	do.....	do.....	2	6.5	35
8.....	June 15.....	Active growth.....	Hours	6	35
9.....	do.....	do.....	3	7	35
92.....	do.....	do.....	3	7	35
10.....	Nov. 11.....	Dormant.....	1	7	35
11.....	do.....	do.....	1	6	35
12.....	Nov. 6.....	do.....	3	7	35
13.....	Nov. 9.....	do.....	3	6	35
14.....	Nov. 12.....	do.....	28	7	35
15.....	Dec. 2.....	do.....	4	8	51
16.....	do.....	do.....	Days	7	46
			10		

Tree No.	<i>C. ulmi</i> isolated from sap displaced at indicated distance above injection point <sup>4</sup>							Upper limit <sup>5</sup> of vascular discoloration	Maximum distance above injection point at which <i>C. ulmi</i> was isolated
	2-5	5-8	8-11	11-14	17-20	24-27	29-32		
1.....	—	—	—	—	—	—	—	Inches	Inches
2.....	—	—	—	—	—	—	—	15	3
3.....	—	—	—	—	—	—	—	16	4
4.....	—	—	—	—	—	—	—	24	2
5.....	—	—	—	—	—	—	—	24	2
6.....	—	—	—	—	—	—	—	18	3
7.....	—	—	—	—	—	—	—	Feet	Feet
8.....	—	—	—	—	—	—	—	29	28
9.....	—	—	—	—	—	—	—	26	26
92.....	—	—	—	—	—	—	—	32	29
10.....	—	—	—	—	—	—	—	30	25
11.....	—	—	—	—	—	—	—	32	30
12.....	—	—	—	—	—	—	—	—	—
13.....	—	—	—	—	—	—	—	(6)	6
14.....	—	—	—	—	—	—	—	(6)	6
15.....	—	—	—	—	—	—	—	(6)	24
16.....	—	—	—	—	—	—	—	(6)	12
								(6)	12

<sup>1</sup> All vessels of the several outer wood rings were injected with spore suspension approximately 18 inches above soil line.

<sup>2</sup> The interval between the beginning of the injection and the time at which the tree was felled and sectioned into 3- or 4-foot lengths.

<sup>3</sup> Measured from the point of injection.

<sup>4</sup> + indicates that *C. ulmi* was isolated; —, that it was not.

<sup>5</sup> Typical yeastlike spores of *C. ulmi* were observed in the sap samples taken from the injected tree sections designated by an asterisk (\*).

<sup>6</sup> No discoloration developed.

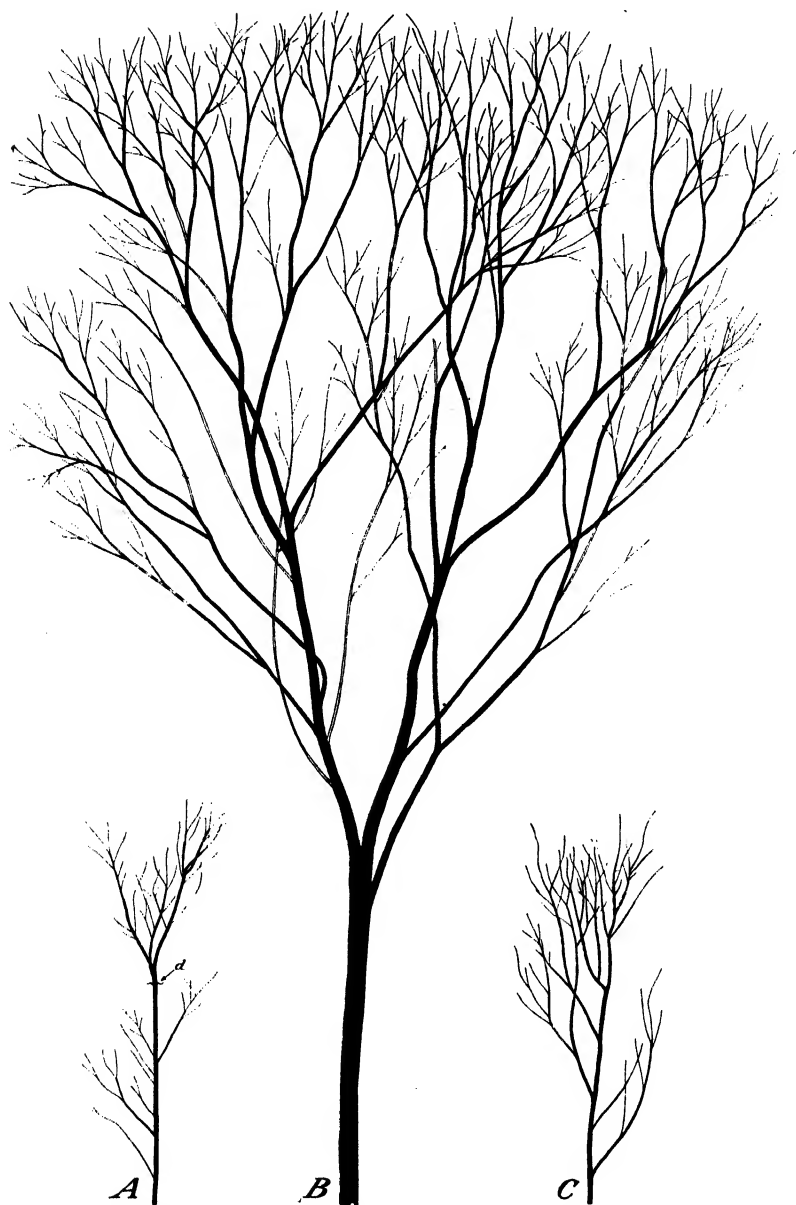


FIGURE 5.—Diagram of spore distribution in American elms: A, Tree injected with a spore suspension of *Ceratostomella ulmi* for 20 minutes at point *d*; B and C, trees injected stump high, B, for 2 hours and 20 minutes, C, for 20 minutes. All trees were felled and defoliated at the close of the injection period. Shading indicates vascular discoloration with which *C. ulmi* was found associated several weeks after injection. Broken shading indicates discontinuous or interrupted streaking of vessels at limits of spore distribution. All trees drawn to scale 0.5 cm.=1 foot. Lower 17 feet of trunk of tree in B not shown.



then centrifuged, its suspended cells being concentrated in a volume of 4 cc. Typical yeastlike colonies of budding spores (fig. 3) were observed in every sap sample collected from tree 9 and in the samples taken from the lower part of the bole and the top or leader branch of tree 8. That most of these cells were spores of *Ceratostomella ulmi* was established by isolation and growth of the spores on acidified malt agar. Four hundred and eighty colonies of *C. ulmi* developed from 1 cc. of the concentrated sample of the sap that had been displaced from a stem section cut from tree 9 at from 17 to 20 feet above the injection point. Eighty colonies per cubic centimeter developed from sample 24-27, tree 9; 200 per cubic centimeter from sample 29-32, tree 9; 3 per cubic centimeter from sample 29-32, tree 8; and an indeterminate high number from all others, except sample 17-20, tree 8, in which no *C. ulmi* colonies could be detected in the mass of contaminating fungi that overran all plates of this series.

TABLE 2.—Distribution of spores of *Ceratostomella ulmi* in small American elms injected stump high in early summer, 1938

Tree No.	Injection date	Spore suspension injected <sup>1</sup>	Tree dimensions		Maximum height <sup>2</sup> of vascular discoloration	Maximum height <sup>2</sup> at which <i>C. ulmi</i> was isolated
			Diameter breast high	Height <sup>2</sup>		
		Cc.	Inches	Feet	Feet	Feet
4 <sup>3</sup>	June 3	125	11 $\frac{1}{4}$	10.4	8.7	8.7
5	do.	200	11 $\frac{1}{2}$	11.1	9.3	9.0
6	June 10	130	11 $\frac{1}{2}$	11.9	9.1	9.1
10	June 22	475	21 $\frac{1}{2}$	13.7	12.3	12.3
11	do.	1,750	31 $\frac{1}{2}$	20.0	19.0	19.0
12	July 17	280	11 $\frac{1}{2}$	11.7	7.5	7.4
13	do.	300	11 $\frac{1}{2}$	11.8	8.3	8.3
14	do.	300	13 $\frac{1}{2}$	13.7	12.2	12.2
15 <sup>4</sup>	do.	450	13 $\frac{1}{4}$	12.1	9.9	9.6

<sup>1</sup> The injection period was 20 minutes to 1 hour in all cases. All injections were made between the hours of 10 a. m. and 3 p. m. on clear days.

<sup>2</sup> Measured from the point of injection.

<sup>3</sup> See figure 5, C.

<sup>4</sup> See figure 6, A.

Confirmation of this rapid upward distribution of spores in these trees during the 3 hours of the injection was obtained from the discoloration that subsequently developed in the outermost or new ring of large spring vessels. This discoloration was well developed within 10 days after injection and extended as a few streaks to 32 and 30 feet, respectively, in trees 8 and 9, and to 32 feet in tree 92, from which the sap was not displaced. Discoloration induced by *Ceratostomella ulmi* was as abundant in the stem sections from which spores were taken by the sap-displacement technique as in those not so treated. *C. ulmi* was recovered from chips of discolored tissue taken from these trees at 29, 25, and 30 feet, respectively, and cultured on agar.

The distribution of *Ceratostomella ulmi* spores in large native elms was studied in more detail by the discoloration procedure. Trees of different height, diameter, and age were injected at the base of the bole by the usual pan and chisel-cut procedure for 20 minutes to 4 hours. Vascular discoloration developed to within a few feet of the tops of most of the trees studied in the early or midseasons of 1937 and 1938. The trees ranged up to 64.8 feet in height. The discolor-

ation in general extended into most of the major branches, into numerous secondary branches, in some instances even into smaller twigs throughout the crowns of the trees. There were major branches in which no vascular discoloration developed, however. In one tree (18 of table 3), the discoloration failed at roughly two-thirds of the height of the tree. With certain minor exceptions, *C. ulmi* was found associated with the vascular discoloration at all levels above the injection points in these trees. On the other hand, all routine attempts to isolate this organism from nondiscolored regions of the vascular system 2 to 5 weeks after injection and felling were negative. Hundreds of such attempts were made over a period of 4 years. The upper limit of spore distribution, as indicated by the discoloration induced, is recorded in table 3. No comparable discoloration (fig. 4, A) developed in the vessels of two 50-foot trees injected with sterile tap water under like conditions, and isolations from the outer vascular rings in these trees did not yield any wilt-inducing fungi.

TABLE 3. - Distribution of spores of *Ceratostemella ulmi* in tall American elms injected stump high in early summer to midsummer, 1937-38

Tree No.	Injection date	Schedule of operations			Spore suspension injected	Tree dimensions		Maximum height <sup>2</sup> of vascular discoloration	Maximum height <sup>2</sup> at which <i>C. ulmi</i> was isolated
		Injection	Felling <sup>1</sup>	Defoliation completed		Diameter breast high	Height <sup>2</sup>		
1937									
100	Aug. 2	Clock time 10:50 a.m.	Clock time 11:55 a.m.	Clock time 12:38 p.m.	Liters 5	Inches 8.5	Feet 38.0	Feet 31.5	Feet 32.2
101	do.	2:00 p.m.	3:00 p.m.	3:40 p.m.	11	9.0	44.0	42.5	34.0
103	Aug. 3	10:40 a.m.	<sup>3</sup> 11:50 a.m.	12:10 p.m.	14	8.5	49.0	45.0	36.0
69	June 9	1:12 p.m.	<sup>3</sup> 4:00 p.m.	10:30 a.m.	20	9.0	50.0	48.0	48.0
70	do.	1:56 p.m.	<sup>3</sup> 3:20 p.m.	11:00 a.m.	14	10.0	50.5	49.0	46.0
71	do.	2:40 p.m.	<sup>3</sup> 3:47 p.m.	11:30 a.m.	14	8.0	50.0	46.2	45.0
73	June 15	11:05 a.m.	<sup>3</sup> 12:00 m.	1:05 p.m.	10.5	9.0	50.0	47.7	47.0
95	July 28	12:30 p.m.	1:30 p.m.	3:10 p.m.	20	9.5	58.0	44.0	39.0
68	June 7	2:38 p.m.	3:40 p.m.	4:15 p.m.	20	10.0	60.0	57.5	53.0
94	July 28	10:30 a.m.	11:30 a.m.	12:25 p.m.	14	8.0	60.0	54.0	53.0
99	July 30	12:30 p.m.	1:30 p.m.	2:00 p.m.	33	12.0	64.0	56.0	51.0
107	Aug. 9	11:30 a.m.	1:30 p.m.	4:00 p.m.	42	12.5	64.8	63.7	63.7
1938									
18	June 30	2:58 p.m.	5:01 p.m.	6:25 p.m.	27	9	53.0	37.5	37.0
19	do.	4:00 p.m.	8:00 p.m.	8:30 p.m.	6	6	49.1	47.2	45.0
43	do.	9:22 a.m.	<sup>3</sup> 11:22 a.m.	12:00 m.	8	8	55.5	54.0	54.0
44	do.	9:39 a.m.	<sup>3</sup> 11:45 a.m.	12:15 p.m.	14	6	53.3	46.8	46.0
45 <sup>4</sup>	do.	12:17 p.m.	<sup>3</sup> 2:37 p.m.	3:10 p.m.	11	6	50.0	47.0	46.5
46	do.	12:50 p.m.	<sup>3</sup> 2:45 p.m.	3:40 p.m.	12	9	52.0	49.6	49.0

<sup>1</sup> The hour at which the injection pan was removed and felling operations were begun.

<sup>2</sup> Measured from the point of injection.

<sup>3</sup> All trees were sectioned at roughly  $\frac{1}{4}$ ,  $\frac{3}{4}$ , and  $\frac{5}{6}$  of their height after felling, except these trees. These were left intact until examination for discoloration was made several weeks after injection.

<sup>4</sup> See fig. 5, B.

The distribution of spores in tree 45, representative of the large-tree class, is diagrammed in figure 5, B. The heavy shading in trunks and branches indicates the presence of more or less continuous streaks of discoloration induced by the spores injected in the outer vascular ring. In most cases these streaks disappeared in the distal parts of the branches. There, isolated points or dashes of color were usually found separated by a few millimeters to decimeters along the vertical axis of the branch. Such a discoloration pattern in the outer ring of large vessels is indicated by broken shaded lines in the diagram.

Representative areas of this discoloration in the outer vascular ring of trunk and crown were cultured. *Ceratostomella ulmi* was consistently isolated from all such areas, including the isolated spots and dashes of color in the vessels at or below the points where the discoloration disappeared. *C. ulmi* was not recovered in any section in these trees where there was no discoloration in the outer vascular ring.

Further details as to the extent and locus of spore distribution in these trees are illustrated by representative tree 107 in figures 6, *B*; 7; and 8.

Most of the vessels of the outer ring in one of the arms of this twin-leadered tree were discolored at 20, 30, and even 40 feet above the injection point (fig. 8, *E, C, A*). Relatively few vessels were discolored in the other arm (fig. 8, *B, D, F*), but again there were roughly as many vessels discolored in it at 40 as at 20 feet above the injection point. The extent of the discoloration in branch *G*, one of the terminal branches of coleader *E, C, A*, is portrayed in the photodiagram (fig. 6, *B*). Its base point was 52 feet above the point of injection. Discoloration extended into all of its secondary branches, commonly into twigs three-eighths of an inch or less in diameter. Photographs of the discoloration induced in the vessels of this branch were taken at points indicated by letters (fig. 7). The highest point to which discoloration extended was 63 feet 8 inches above the injection point, 13 inches below the end of this branch. The branch at this point was 3 years old and three-sixteenths of an inch in diameter. *Ceratostomella ulmi* was isolated at many representative points throughout the trunk and crown, even to the uppermost limits of the discoloration indicated in figure 6, *B*. Tree 107, injected at 11:30 a. m., August 9, 1937, took up 42 liters of spore suspension of approximately 3,000,000 spores per cubic centimeter within 1 hour. The injection ended at 12:30 p. m. The tree was felled at 1:30 p. m., and was sectioned at 20, 40, and approximately 50 feet above the injection point by 2 p. m. No discoloration was present at the points sectioned at this time. All branches were defoliated by 4 p. m. It was a codominant member of a close stand of hardwoods on poorly drained soil.

#### UPPER LIMIT OF SPORE DISTRIBUTION

The distribution of spores that occurred in trees felled in early summer or midsummer, within 20 minutes to 24 hours after injection, generally terminated above the injection point in the same type of growth, regardless of the height of the elms injected (fig. 6) or the height at which injection was made (fig. 5). Thus in tree 107, vascular discoloration from which *Ceratostomella ulmi* could be isolated generally stopped in the terminal branches a few inches or feet below the terminal growth scars formed in the preceding season. This was as much as 63.7 feet above the injection point in this 64.8-foot tree. The branches at these points were 2 to 10 years old, but generally 3 years old or more. In diameter they varied considerably, but usually they were one-eighth to three-eighths of an inch. The fungus was not distributed into all such, to be sure, since some branches, even an occasional major branch, developed no vascular discoloration in this and other trees.



FIGURE 6.—Photodiagram of spore distribution in representative trees injected stump high for 1 hour with spore suspension of *Ceratostomella ulmi*. Shading indicates vascular discoloration with which *C. ulmi* was found associated several weeks after injection; broken lines indicate parts of branching structure where discoloration was not produced and from which *C. ulmi* could not be isolated. Trees felled and defoliated at close of injection period. A, Small tree (No. 15) shown entire. B, Branch G, from top of 64.8-foot tree 107, excised at 52 feet above point of injection, at close of injection period. Photographs of discoloration induced in vessels, taken at the points indicated by lower-case italic letters, are presented in figure 7. For extent of vascular discoloration at points below 50 feet in this branch, see figure 8, A, C, and E.

Spores injected July 23, 1937, at 41 feet above the ground in 67-foot tree 86 of table 7 were distributed throughout that portion of the crown supported by the axis injected. The vascular discoloration from which the injected fungus could be recovered terminated in the

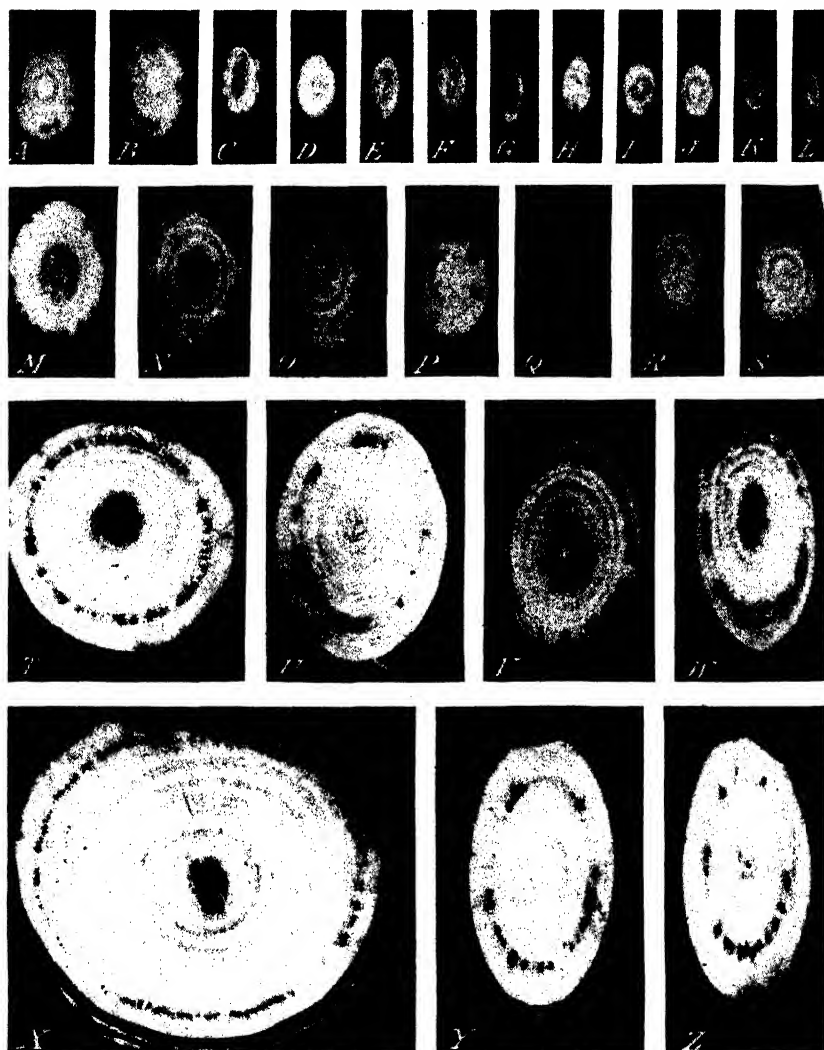


FIGURE 7.—Discoloration induced by injected spores in ring of vessels of current season in branch G, tree 107, as seen in cross sections taken at points indicated by letters as in figure 6, B.

same kind of growth 2 feet or more from the 1936 terminal growth scars. In tree 42 of table 7, injected 41 feet above the stump June 28, 1938, it extended in certain cases to within a few inches of the branch tips.

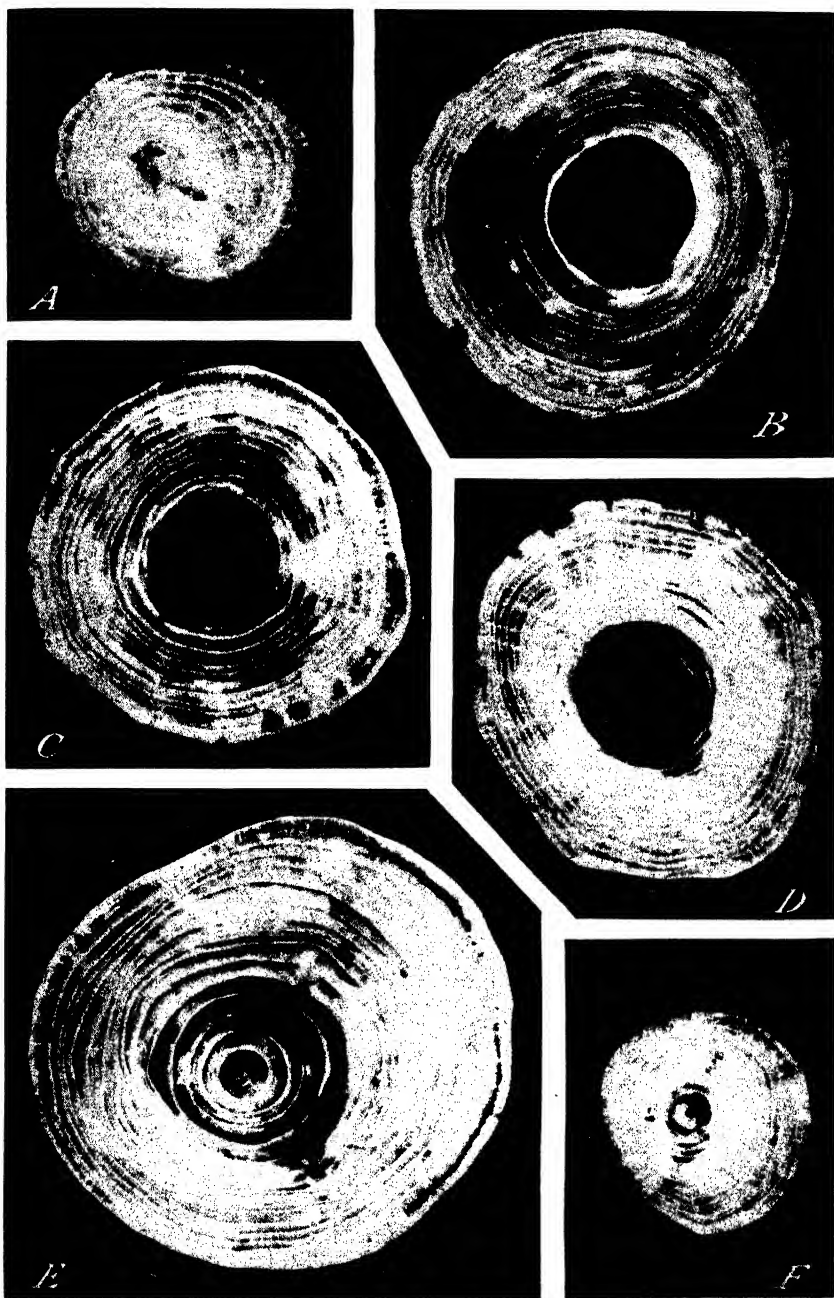


FIGURE 8.—Discoloration induced by *Ceratostomella ulmi* in the new ring of vessels of tree 107 as seen in representative cross sections taken from the two main branches at 20 feet (B, E), 30 feet (C, D), and 40 feet (A, F) above injection point.  $\times \frac{1}{2}$ .

Spores injected into terminal branches 18 to 29 feet above the stump in American elms, at points where the diameter of these branches did not exceed one-half inch, were distributed to within about the same minimum distance from the branch ends as they were in injections made at the base of the crown or lower. Discoloration ended in these terminal branches at or several inches below the terminal growth scars formed at the close of the preceding season. The frequency with which discoloration extended to such points was higher in these terminal-branch injections than in injections made at lower levels.

Vascular discolorations from which *Ceratostomella ulmi* can be isolated, however, in time generally extend into the terminal growth produced during the season of injection. Thus, in the type of injection just described it was commonly present in the current season's growth of the branch terminals 5 days after injection. However, it generally failed in these branches at about 12 inches from the apices of the new growth. Two weeks after injection it still failed generally at about the same distance from the apices of the new growth. Discoloration at this time, however, did extend in one case to the end of such growth in a terminal branch. By the end of the season it was commonly found to the limits of growth throughout the crowns of trees injected before midsummer, but even then it frequently came to an end below the terminus of the growth produced during the season of injection. Although most of the branches and twigs in the crowns of such trees were then invaded by the fungus, there were notable exceptions wherein even relatively large secondary branches were found without discoloration or invasion by the fungus.

#### TREES INJECTED IN LATE SUMMER OR EARLY AUTUMN

Spores of *Ceratostomella ulmi* were injected by the usual procedure at stump height into elms of different height in the latter part of the seasons of 1937 and 1939. In general the distribution of spores in these trees was markedly different from that in trees injected in the early part of the season. The locus of distribution was the same, i. e., typical discoloration developed only in the outer ring of large spring vessels, but the distance to which the spores were distributed above the injection point was conspicuously limited as compared with that in trees injected in the early part of the season. Thus, spores of *C. ulmi* apparently were not distributed to roughly more than one-half the height of 10 small trees (less than 25 feet in height) injected in the latter half of the 1937 season (table 4), whereas in the 9 small trees studied in the early half of the 1938 season (table 2) they rose to within an average distance of 2.2 feet of the tips of trees that averaged 12.9 feet in height. The records of spore distribution in tall trees up to 64.8 feet in height display the same trend, but in more striking contrast. The average height of the 18 tall trees injected with spores early in the seasons of 1937 and 1938 (table 3) was 52.8 feet; the average height of spore distribution was 48.3 feet, judged from vascular discoloration induced by the injected spores. The average height of spore distribution in 15 large (greater than 26 feet in height) leafy trees injected in early autumn in 1937 and 1939 was 12.3 feet; the average height of the trees was 38.1 feet. In other words, spores were not distributed to more than an average of three-tenths of the height of trees injected in late season, whereas they were distributed to more than nine-tenths of the height of the trees injected in early season.

TABLE 4.—*Distribution of Ceratostomella ulmi spores in American elms injected stump high in late summer and early fall, 1937 and 1939*

Tree No.	Injection date	Injection time	Spore suspension injected	Tree dimensions		Maximum height <sup>1</sup> of vascular discoloration	Maximum height <sup>1</sup> at which <i>C. ulmi</i> was isolated
				Diameter breast high	Height <sup>1</sup>		
	1937	Hours	Liters	Inches	Feet	Feet	Feet
105	Aug. 3	1	3.0	2.0	13.5	6.5	4.0
104	do	1	1.2	2.5	16.0	4.5	4.0
119	Sept. 23	1	2.0	2.0	15.0	6.8	( <sup>2</sup> )
120	do	1	1.5	2.5	13.0	6.7	4.0
121	do	1	1.5	2.0	14.0	7.5	4.0
126	Oct. 8	1	1.5	2.0	15.0	7.5	6.0
127	do	1	2.5	3.0	18.3	8.0	7.5
102	Aug. 3	1	4.0	4.0	21.0	9.0	8.0
108	Aug. 11	1	8.0	3.0	21.3	8.0	8.0
117	Aug. 23	1	3.0	4.0	23.0	21.5	( <sup>3</sup> )
128	Oct. 8	2	4.0	4.0	27.0	11.0	11.0
129	do	2	6.0	5.0	32.0	9.0	9.0
123	Oct. 2	3	5.0	4.0	39.0	10.0	9.0
124	do	2	6.0	5.0	45.0	12.0	11.0
122	Sept. 24	2	40.0	10.0	57.0	29.0	29.0
125	Sept. 30	2	89.0	20.0	60.0	30.3	25.0
	1939						
973	Sept. 28	2.0	3.5	4.2	32.5	12.0	12.0
974	do	1.0	4.0	4.5	34.2	12.0	12.0
975	do	1.8	4.6	5.5	37.1	7.3	7.3
976	Oct. 5	<sup>4</sup> 4.0	5.0	4.0	28.5	11.0	11.0
977	do	<sup>4</sup> 4.2	7.0	5.0	35.2	9.8	<sup>5</sup> 9.0
978	do	<sup>4</sup> 4.2	2.9	3.0	28.6	8.2	<sup>5</sup> 8.0
979	do	<sup>4</sup> 3.7	7.3	6.3	39.7	8.6	<sup>5</sup> 8.0
980	do	<sup>4</sup> 4.0	6.3	4.0	39.8	7.7	<sup>5</sup> 7.0
981	do	4.0	4.7	3.7	36.2	6.7	<sup>5</sup> 6.0

<sup>1</sup> Measured from the point of injection.<sup>2</sup> No isolations were made from this tree.<sup>3</sup> All attempts to isolate *C. ulmi* from the vascular discoloration recorded failed.<sup>4</sup> The injection pan was empty when the tree was felled at the end of the period indicated. The exact length of the injection is therefore unknown.<sup>5</sup> No isolation was attempted at the maximum height to which vascular discoloration extended.

The number of vessels invaded within a few feet of the point of injection in trees injected in the latter part of the leafy season was essentially the same as in trees injected in early season, that is, practically all vessels of the outer ring were invaded and a solid ring of discoloration was produced; but, whereas this more or less complete ring of discoloration usually continued for many feet above the injection point in large trees injected in early season (figs. 7 and 8), the number of discolored vessels rapidly diminished with increasing distance above the injection point in trees injected in the latter part of the season. Thus, in 57-foot tree 122 (table 4), typical of those studied in early autumn, there was a solid ring of discoloration involving practically all vessels of the ring at 2 feet above the injection point. At 5 feet above the injection point there were 496 spots of discoloration, evenly distributed through the ring, each spot involving 1 to several vessels. Ten feet above the injection point there were but 97 such spots; at 20 feet, only 5; above 25 feet, only 1.

There appears to be no definite time at which this transition occurs. The results for spore distribution in trees injected in the early season were consistent (tables 1, 2, and 3), as were those for trees injected in late season (table 4), but those injected in late July or early August, i. e., in midseason, were highly variable. For convenience, the results from trees injected in this transitional period were tabulated either with those from trees injected in early season or with those



from trees injected in late season, according to their conformation to the one group or the other.

#### TREES INJECTED DURING DORMANT PERIOD OF LATE AUTUMN OR EARLY WINTER

Spores of *Ceratostomella ulmi*, when injected stump high into American elms after leaf fall in the autumn, could not be recovered at more than 24 inches above the point of injection. None were detected in sap displaced from sections of the injected stem taken at 2 to 5 feet above the injection point, nor elsewhere throughout these trees. No discoloration subsequently developed in the new ring of vessels or elsewhere in these injected trees, even though the logs into which they were cut were held in the greenhouse at approximately 60° F. until March of the following year. In every case, however, the organism was isolated at from 6 to 24 inches above the point of injection (table 1) by the following procedure. Sections of stem 6 inches in length were taken from the injected trees at respectively  $\frac{1}{2}$ , 1, 2, and 5 feet above the injection point. Chips to a depth of several rings were taken aseptically from the complete circumference of these sections and cultured on potato-dextrose agar. The cultures were held at room temperature and were checked for the presence of *C. ulmi* 7 and 21 days later.

#### GIRDLED TREES INJECTED DURING SUMMER

Spores of *Ceratostomella ulmi*, injected several hours by the usual pan and chisel-cut procedure into two girdled leafy trees, were not distributed more than 16 inches above or below the point of injection. The trees had been girdled the preceding February by application of a band of copper sulfate around the trunk by a technique described by Liming<sup>6</sup> in 1937. These trees consequently produced during the season of injection no new ring of large vessels for a distance of several feet above or below the girdle. The injection of spores was made near the upper limits of the girdled zone. The trees were felled at the close of the injection period. The several outer growth sheaths of stem tissue were generally colored a deep brown by the copper sulfate in the region of the chemical girdle. No vascular discoloration was induced in these discolored areas of tissue by the injected spores. The injected spores, however, induced discoloration in many of the small vessels of all the outer rings severed by the chisel cuts that had not been discolored by the chemical girdle. This discoloration did not extend in these vessels beyond 16 inches. The fungus was recovered from the discolored vessels up to the limit of discoloration, i. e., 16 inches.

#### SPORES OF DOTHIORELLA (CEPHALOSPORIUM) ULMI AND VERTICILLIUM DAHLIAE

Spore suspensions of *Dothiorella ulmi* and *Verticillium dahliae* were apparently distributed after the manner described for suspensions of spores of *Ceratostomella ulmi* when injected into the bases of trees early in June 1936. The injections were made stump high by the usual procedure. Several cubic centimeters of concentrated spore suspension was introduced into each tree at each of three points around

<sup>6</sup> LIMING, O. N. THE DUTCH ELM DISEASE ERADICATION PROGRAM—OBJECTIVE, METHODS, AND RESULTS. U. S. Dept. Agr., Plant Dis. Rprtr. Sup. 99: 18-25. 1937. [Mimeographed.]

the circumference. The width of the chisel cuts was 1 inch. The locus and limits of spore distribution were determined from discoloration seen after stripping off the bark when the trees were felled 6 or 7 days later. The discoloration in each instance began at the injection points at the base of the trees and extended up the stem as a series of continuous streaks for various distances, after which the streaks continued in an interrupted pattern—short streaks intercepted by progressively greater distances of clear tissue—until all color disappeared. These fungi did not induce vascular discolorations differing sufficiently to be diagnostic. The discoloration induced by *C. ulmi* was of the same general appearance, type, and color for each organism, i. e., a red to greenish brown of varying intensity, strictly limited to the functional ring of large vessels. It extended to within 1 to 3 feet from the tops of the trees inoculated in this manner with *V. dahliae* and *C. ulmi*, and disappeared at 8 to 11 feet below the tops of trees injected with *D. ulmi* (table 5). The organism injected was recovered in all instances at the limits of discoloration above the injection points and at other representative points in isolations made at the time the vascular discoloration was traced. Only from the trees injected with *C. ulmi* was the injected organism recovered beyond the limits of discoloration. Field observations of these diseases had suggested, however, that the pathogens involved are distributed downward as well as upward, by spores rather than by continuous mycelial growth. The following experiments were therefore undertaken to ascertain whether spores could be distributed downward in elms at various seasons.

TABLE 5.—Distribution of spores of *Verticillium dahliae*, *Dothiorella ulmi*, and *Ceratostomella ulmi* in American elms, injected<sup>1</sup> stump high with spore suspensions of these fungi, in June 1936

Tree No.	Organism injected	Time between injection and examination	Tree dimensions		Maximum height <sup>2</sup> of vascular discoloration	Maximum height <sup>2</sup> at which pathogen was isolated
			Diameter breast high	Height <sup>2</sup>		
		Days	Inches	Feet	Feet	Feet
V6	<i>V. dahliae</i>	6	5	32	30	30
V7	do	6	5	28	27	27
V8	do	6	4	23	21	21
D52	<i>D. ulmi</i>	7	6	44	33	33
D53	do	7	6	44	33	33
D54	do	7	6	40	32	32
C1	<i>C. ulmi</i>	7	7	35	32	32
C2	do	7	6	30	26	29
C3	do	7	6	31	29	30

<sup>1</sup> A few drops of spore suspension were injected into these trees by the usual procedure at one to three points distributed around the circumference of the trunk at one horizontal level 4 to 18 inches above the soil line.

<sup>2</sup> Measured from the point of injection.

## DISTRIBUTION OF SPORES OF CERATOSTOMELLA ULMI IN TOP-INJECTED ELMS

### INJECTION OF LARGE VOLUMES OF SPORE SUSPENSIONS INTO MAIN STEMS AT BASE OF CROWNS

Large volumes of spore suspensions of *Ceratostomella ulmi* were injected into the main stem of tall polelike elms 13 to 41 feet above the stump at various seasons of the year. The spores were injected through chisel cuts made under a spore suspension held around the

trunk with cone-frustrum pans. All functional vessels in the new-growth sheath at the pan level were injected. The stems in most cases were then cut off some 6 to 10 inches above the pans immediately after the injection was concluded. At varying intervals of time the trees were cut down and sectioned. The maximum distribution of the fungus was then determined by the methods earlier described.

During a 3-day injection period prior to leaf development in the spring, the maximum distribution of the fungus below the injection point was 2 feet. Spores were not recovered. Discoloration, however, developed in the three spore-injected trees. The fungus was isolated from this discoloration at 2, 2, and 1 feet, respectively, below the injection points (table 6).

Spores were recovered at greater distances below the injection points in dormant trees during late autumn and early winter (table 6). The maximum time during which the trees under investigation absorbed the spore suspension probably did not exceed 12 hours; i. e., the injections were initiated at noon, in the case of trees 14, 15, and 16, and the pans were kept supplied with spore suspension until twilight, after which no further additions of spore suspension were made. When the injection was continued for 3 hours or longer, the spores were consistently recovered at 8 to 12 feet below the injection points. There was apparently no further distribution of spores in these trees with the passage of time. Thus, spores were recovered at 8 or 12 feet below the injection points, on trees 12 and 13, 3 hours after the inception of injection. Four or 14 days after the beginning of injection the spores apparently had not been distributed any farther and were recovered again only at 8 and 12 feet, respectively, below the injection points.

No discoloration developed in these trees during observations made between December 2 and January 15, even though the stem sections were held at about 60° F. in the greenhouse. Confirmation of the limits of distribution of the spores was obtained in each instance, however, by culturing chips from the complete circumference of the stem at various distances below the injection point.

*Ceratostomella ulmi* was recovered from the stem at the ground level within 2 days after top injections of a spore suspension of the fungus into trees during the leafy season, within a few weeks of the time of autumn defoliation. The injections were made at 21 to 34 feet above the stump (table 6). Injection in most instances was initiated approximately at noon. As fast as the spore suspension was consumed during the afternoon, the pans were replenished with additional quantities. At twilight the pans were filled for the last time. The spore suspension was absorbed, as were also dye solutions (discussed in a later section) at the rate of 1 to 2 liters per hour between the hours of noon and 6 p. m. Spores were not recovered in the early (September) trials. Discoloration of the vessels and subsequent recovery of the fungus by culture demonstrated, however, that spores had been distributed to distances of 10 and 19 feet below the point of injection in these trees (24 and 20 of table 6). Spores were recovered in subsequent trials. They were observed under the microscope in the displaced sap in a number of instances. Twenty-four hours after injection they were recovered 13 and 17 feet below injections made at 24 and 25 feet,

TABLE 6.—*Distribution of spores of Ceratostomella ulmi below points of injection in the tops of American elms, 1936*

Tree No.	Injection date	Seasonal condition of trees	Time between injection and sectioning <sup>1</sup>	Tree dimensions	
				Diameter breast high	Height <sup>2</sup>
			Days	Inches	Feet
17	May 1	Leaves unfurling	3	4	20
18	do	do	3	4	20
19	do	do	3	4	20
			Hours		
20	Sept. 17	Prior to autumn leaf fall	17	9	25
21	Oct. 5	do	20	6	21
22	do	do	24	7	25
23	Oct. 13	do	24	8	24
24	Sept. 23	do	42	9	29
25	Oct. 7	do	46	7	27
26	Oct. 13	do	46	8	25
			Days		
27	Sept. 23	do	5	6	24
28	do	do	5	6	24
29	Oct. 5	do	14	9	34
30	Sept. 23	do	21	9	34
			Hours		
10	Nov. 11	Dormant	1	7	13
11	do	do	1	6	13
12	Nov. 6	do	3	7	13
13	Nov. 9	do	3	6	23
14	Nov. 12	do	27	7	23
			Days		
15	Dec. 2	do	4	8	22
16	do	do	14	7	24

Tree No.	<i>C. ulmi</i> isolated from sap displaced at indicated distance (feet) below injection point <sup>3</sup>						Lower limit of vascular discoloration	Maximum distance below injection point at which <i>C. ulmi</i> was isolated from discolored vessels
	1-3	4-7	7-10	10-13	13-17	17-21	21-25	
								Feet
17	—	—	—	—	—	—	—	2
18	—	—	—	—	—	—	—	2
19	—	—	—	—	—	—	—	1
20	—	—	—	—	—	—	—	23
21	—	+	+	—	—	—	—	21
22	—	+	+	+	+	—	—	25
23	—	+	+	+	+	—	—	23
24	—	+	+	+	+	—	—	18
25	—	+	+	+	+	+	+	27
26	—	+	+	+	—	+	—	25
27	—	—	—	—	—	—	—	24
28	—	—	—	—	—	—	—	20
29	—	—	—	—	—	—	—	34
30	—	—	—	—	—	—	—	34
								Feet
	1-4	4-8	8-12	12-16				
10	+	—	—	—			(1)	4
11	+	—	—	—			(1)	5
12	+	+	+	—			(1)	12
13	+	+	+	—			(1)	8
14	+	+	+	—			(1)	8
15	+	+	+	—			(1)	12
16	+	+	—	—			(1)	8

<sup>1</sup> The interval between the beginning of the injection and the time at which the tree was felled and sectioned.

<sup>2</sup> All vessels of the several outer rings were injected with spore suspension at the height above the stump indicated. The diameters of the stems at the point of injection ranged from 2 to 5 inches.

<sup>3</sup> + indicates that *C. ulmi* was isolated; —, that it was not. Typical yeastlike spores (fig. 3) of *C. ulmi* were observed in the sap samples taken from the injected tree sections designated by an asterisk (\*).

<sup>4</sup> No discoloration developed.

respectively, above the stump; 46 hours after injection they were observed and identified at 25 feet below an injection made 27 feet above the stump.

Several weeks after the trees had been cut down, vascular discoloration was found to be abundant within several feet of the injection point; this discoloration became progressively less with increasing distance below the injection point; in most instances it finally failed as a few scattered streaks or a single one at the limits indicated (table 6). On the stem of tree 25, for example, several hundred streaks (dots in the outer spring vessel ring in transverse sections) were present 13 feet below the injection point; 54 were present at 20 feet, 33 at 24 feet, and 5 at the stump, or 27 feet below the injection point. *Ceratostomella ulmi* was consistently isolated from all 5 streaks in the stump and from streaks at other points from which recovery was attempted in this tree. In the stem of tree 22, 20 streaks developed at 17 feet below the injection point, 7 at 20 feet, and only 1 at 25 feet below. *C. ulmi* was isolated at all points from which recovery was attempted, except at 24 feet.

Several trees that were top-injected were not examined or cut down until 5 to 21 days after injection. Discoloration was detected in the outermost spring vessels of the stem of tree 28, 5 days after injection. It was steel blue in color and of rather low intensity. Within 10 feet of the injection point it was much more conspicuous by virtue of the fact that many vessels were discolored. About a month later a final examination revealed the usual deep-brown discoloration in the vessels through which spores of *Ceratostomella ulmi* had been distributed. Two streaks extended to 20 feet (4 feet above the stump) in tree 28 (table 6). *C. ulmi* was recovered from both streaks within an inch of their termini. Two typical streaks, one involving four or five vessels, extended into the stump 24 feet below the injection point in tree 27. *C. ulmi* was recovered from both at 20 feet, but not at 24 feet, below the injection point. Six streaks of discoloration, each embodying one to several vessels, were found in the stump 34 feet below the injection point in tree 29, which was cut down 2 weeks after injection. *C. ulmi* was recovered from each of the four streaks cultured. Trees cut at 3 and 4 weeks, respectively, after injection yielded like results (table 6).

Spores of *Ceratostomella ulmi* were injected at the top of the bole in several small and large trees during the early half of the leafy season. The spores were injected by the pan and chisel-cut procedure for 15 minutes to 1 hour. The trees were then felled. The large trees were cut into 10- to 20-foot logs; the small ones were not sectioned. All were defoliated within 2 hours after felling. The tops of the large trees were not cut off until after felling.

One month after injection, discoloration induced by the spores extended in the outer ring of vessels to the stump (fig. 5, A) in all trees injected directly with spore suspension (table 7). In trees 1, 2, and 3, respectively, 39, 5, and 5 vessels of the new ring were discolored by the spores at the stump, or roughly 7 feet below the injection points in the smaller trees. In the 2 tall trees injected directly with spore suspension, 15 and 20 of these vessels were discolored at the stump, 40 and 41 feet, respectively, below the injection points. Only 1 streak of discoloration was found at the lower limits of spore distribution 32 and 32.5 feet, respectively, below the point of injection in the 2 tall trees that were injected for 1 hour with sterile water prior to

spore injection. Each spot or streak of discoloration in the ring was interpreted as originating in 1 vessel. Several vessels may have been involved, however. *Ceratostomella ulmi* was recovered at representative points from these discolored vessels in all cases.

TABLE 7.—Distribution of *Ceratostomella ulmi* from points at which spore suspension was injected into elms near the top of the main stem

Tree No.	Injection date	Tree dimensions			Schedule of operations			Liquid absorbed	
		Diameter breast high	Height	Diameter at injection point	Water injected	Spore suspension injected	Tree felled	Water	Spore suspension
		Inches	Feet	Inches	Clock time	Clock time	Clock time	Liters	Liters
85 <sup>1</sup> .....	1937 July 23	9	61.0	4.5	.....	2:12 p. m.	2:27 p. m.	.....	3
86.....	do	12	67.0	5.0	.....	10:50 a. m.	11:10 a. m.	.....	6
	1938								
41.....	June 28	10	57.0	3.3	2:25 p. m.	3:25 p. m.	4:25 p. m.	8	6
42.....	do	12	62.2	3.5	10:45 a. m.	11:45 a. m.	12:45 p. m.	16	13
1.....	June 2	1.6	13.5	1.0	.....	10:30 a. m.	10:50 a. m.	.....	.3
2 <sup>2</sup> .....	do	1.2	12.0	.6	.....	10:40 a. m.	11:00 a. m.	.....	.3
3.....	do	1.3	12.4	.7	.....	11:10 a. m.	11:30 a. m.	.....	.3

Tree No.	Stem below points of injection										Stem above point of injection				
	Total length	Vessels discolored at distances indicated (feet) below injection point <sup>3</sup>								Levels below injection point at which <i>C. ulmi</i> was isolated	Total length	Terminus of discoloration			Maximum height above injection point at which <i>C. ulmi</i> was isolated
		5	10	15	20	25	30	35	40			Height above injection point	Stem diameter	Stem age <sup>4</sup>	
85 <sup>1</sup> .....	Feet 40.0	Pct. 75	Pct. 50	Pct. 33	Pct. 25	No. 77	No. 48	No. 18	No. 15	Feet 30, 40	Feet 21.0	Feet 17.0	Inches 1	.....	Feet 17.0
86.....	41.0	50	No. 131	Pct. 98	No. 27	.....	22	20	20	31, 41	26.0	23.0	3/4	.....	23.0
41.....	38.5	75	Pct. 50	Pct. 25	Pct. 50	5	3	0	0	24, 32	18.5	.....	.....	.....	.....
42.....	41.0	95	90	.....	50	.....	3	0	0	20, 25	21.2	20.5	3/16	3	20.5
1.....	8.0	No. 35	No. 39	.....	.....	.....	.....	.....	.....	6, 8	5.5	4.8	3/16	3	4.1
2 <sup>2</sup> .....	6.7	13	5	.....	.....	.....	.....	.....	.....	4, 6.7	5.3	4.5	5/16	3	4.0
3.....	6.7	12	5	.....	.....	.....	.....	.....	.....	4, 6.7	5.7	4.6	5/16	3	4.6

<sup>1</sup> *Ulmus fulva*; all others, *U. americana*.

<sup>2</sup> See fig. 5, A.

<sup>3</sup> Estimates of percentage of total vessels discolored in the outer ring are given where accurate determination was difficult as a result of fusion of many spots of color into a solid band (fig. 8). Each spot was considered as one discolored vessel; actually it usually involved several.

<sup>4</sup> Determined from terminal growth scars. In each case listed the age of the branch at the point indicated was equal to the number of vessel rings present plus 2.

<sup>5</sup> Number of spots of discoloration in outer ring at stump or base of tree.

#### INJECTION OF A FEW DROPS OF SPORE SUSPENSION INTO TERMINAL BRANCHES

Relatively small quantities of spores were introduced into the vessels of terminal branches in injections made through chisel cuts during the dormant season of early spring and in midsummer. The injections were made with a 1/4-inch chisel, 8.6 to 29 feet above the stumps, in terminal branches one-half to 1 inch in diameter. Certain other trees not recorded in the tables and injected in early spring were felled 6 weeks after injection; the distribution of spores was traced at once from discolorations that had been induced in the injected vessels

by the spores introduced. The trees injected in midsummer were felled 1, 5, and 14 days after injection and were immediately defoliated. One month later the trees were examined and the distribution of spores was traced by the discoloration procedure.

The spores injected in this manner April 1, 1937, into 12 dormant trees approximately 40 feet in height did not descend more than 19 inches in 6 weeks. When the trees were felled May 11, the maximum extension of discoloration in the outermost ring of large vessels injected was 8 inches above and 19 inches below the injection point. The average distribution in these vessels below the injection point was 5.4 inches; the average distribution above, 1.4 inches. *Ceratostomella ulmi* was isolated from the discolored vessels of the 1936 ring at representative points above and below the injection points.

TABLE 8.—Distribution of *Ceratostomella ulmi* 24 hours after spore injections made in chisel cuts July 29, 1937, into the terminal branches of American elms

Tree and branch No.	Tree dimensions		Injection point		Stem below injection point							
	Diameter breast high	Height	Stem diameter	Stem age	Total length	Vessels discolored † at indicated distance (feet)						
						1	2	3	6	9	12	16
	Inches	Feet	Inch	Years	Feet	No.	No.	No.	No.	No.	No.	No.
9-1	3.8	27.5	3 1/2	5	24.0	16	12	9	5	6	2	1
13-1	2.0	18.5	3 1/2	5	13.2	16	8	7	1	0		
14-1	2.8	21.8	3 1/2	5	17.3	1	1	0				
14-2			3 1/2	6	17.7	4	3	1	0			
15-1	3.3	20	3 1/2	5	14.5	4	3	1	0			
15-2			3 1/2	5	8.6	16	13	9	1	0		
16-1	3.0	18.3	3 1/2	5	14.8	13	9	7	3	0		
16-2			3 1/2	5	14.8	5	4	5	5	2	0	
17-1	2.5	22	3 1/2	4	18.0	2	2	1	0			
17-2			3 1/2	4	18.0	8	8	8	5	2	1	0

Tree and branch No.	Total length	Stem above injection point						Terminus of discoloration		
		Vessels discolored † at indicated distance (inches)						Stem diameter	Stem age	Distance from tip of branch
		6	12	18	24	36	48			
	Inches	No.	No.	No.	No.	No.	No.	Inch	Years	Inches
9-1	43	12	3	1	0			3 1/2	3	25
13-1	64	19	11		6	1	0	3 1/2	2	28
14-1	53	3	1	0				3 1/2	3	41
14-2	48	3	3	2	1	0		3 1/2	3	24
15-1	66	13			5		1	3 1/2	2	22
15-2	71		8		2		1	3 1/2	2	23
16-1	41		5		1	0		3 1/2	2	29
16-2	41	3	1	1	0			3 1/2	3	23
17-1	48	1	1	0				3 1/2		36
17-2	42	13	8	2	2	0		3 1/2	2	28

† *C. ulmi* was consistently isolated at representative points from the discolored vessels in practically all trials made.

Spores injected in this manner at 37 points into the branches of 20 leafy trees 18 to 34 feet in height July 29, 1937, apparently descended as much as 16 feet in 24 hours and to the base of some trees in less than 5 days. A varying number of the vessels injected by the chisel injury were discolored in the 10 treated branches of 6 trees felled 24 hours after injection (table 8). Vessels not injected by the chisel injury were not discolored nor could *Ceratostomella ulmi* be isolated from them. *C. ulmi* was isolated with ease from the discolored vessels at representative points. The minimum distribution of spores as indicated by discoloration in the trees felled 24 hours after injection

was 1 foot, in tree 14-1 (table 8). The maximum distribution was 16 feet, in tree 9.

TABLE 9.—Distribution of *Ceratostomella ulmi* 5 days after spore injections made in chisel cuts July 29, 1937, into the terminal branches of American elms

Tree and branch No.	Tree dimension		Injection point		Stem below injection point								
	Diameter, breast high	Height	Stem diameter	Stem age	Total length	Vessels discolored <sup>1</sup> at indicated distance (feet)							
						1	3	6	9	12	15	18	29
	<i>Inches</i>	<i>Feet</i>	<i>Inch</i>	<i>Years</i>	<i>Feet</i>	<i>No.</i>	<i>No.</i>	<i>No.</i>	<i>No.</i>	<i>No.</i>	<i>No.</i>	<i>No.</i>	<i>No.</i>
2-1	2.5	22.5	$\frac{3}{8}$	6	18.5	2	1						
2-2			$\frac{1}{8}$	5	18.0	2	0						
3-1	1.8	20.3	$\frac{1}{2}$	5	15.5	$2 \pm 50$	$\pm 50$	22	18	18	16		
6-1	4	33.2	$\frac{1}{2}$	3	29.0	21	20	20	( <sup>3</sup> )				
6-3			$\frac{1}{2}$	4	29.0	16	15	12		36			
6-2			$\frac{1}{2}$	6	24.0	20	16	22		20	41	23	6
18-1	2.5	17.7	$\frac{1}{2}$	3	11.7	15	15	15					
18-2			$\frac{1}{2}$	3	11.2	$\pm 26$	20		15	3			
18-3			$\frac{3}{8}$	3	12.5	$\pm 35$	33	16					
19-1	3.5	22.3	$\frac{5}{8}$	4	18.3	14	14	8					
19-2			$\frac{3}{8}$	3	18.8	20	11	8	50			35	
19-3			$\frac{1}{2}$	3	18.8	$\pm 48$	40	37					

Tree and branch No.	Total length	Vessels discolored <sup>1</sup> at indicated distance (inches)					Terminus of discoloration		
		12	18	24	36	48	Stem diameter	Stem age	Distance from tip of branch
		<i>Inches</i>	<i>No.</i>	<i>No.</i>	<i>No.</i>	<i>No.</i>	<i>Inch</i>	<i>Years</i>	<i>Inches</i>
2-1									
2-2	54	$2 \pm 30$		24	10	0	$\frac{3}{8}$	1	10
3-1	58	28		19		2	$\frac{1}{8}$	1	7
6-1	42	5	4	1	0		$\frac{1}{2}$	1	15
6-3	50	5	4	1	0	0	$\frac{1}{2}$	2	18
6-2	55	11			6	1	$\frac{1}{2}$	2	7
18-1	48	$\pm 25$		$\pm 20$	3	0	$\frac{3}{16}$	1	8
18-2	68	8	5	5	4	0	$\frac{3}{16}$	2	27
18-3	46	$\pm 12$	10	8	0		$\frac{3}{16}$	1	11
19-1									
19-2	66	16	16	16	10	3	$\frac{3}{16}$	2	22
19-3									

<sup>1</sup> *C. ulmi* was consistently isolated at representative points from the discolored vessels in practically all trials made.

<sup>2</sup> Fusion of the spots of discoloration made accurate determination of the number of vessels involved difficult.

<sup>3</sup> The branches fused into a single stem between the points indicated by the braces; for example, branches 1 and 3 of tree 6 fused 7 feet below the points of injection.

Discoloration was present to the ground line in 4 of the 5 trees felled 5 days after injection. The fungus had gained access by this time to many vessels all around the stem, most of which had not been injected. An indeterminable number of vessels were discolored 1 foot below the injection points in 4 of the 12 leader branches injected in these trees. The maximum number of vessels discolored at the ground level was 35, in tree 19; the maximum distance of spore distribution, as indicated by discoloration, was 29 feet, or to the stump, in the tallest tree examined at this time, tree 6 (table 9). Only two vessels were discolored below the injection point in each of two leaders injected in tree 2. Six feet was the maximum downward distribution of discoloration in this 22.5-foot tree injected 18 feet above the stump.

Two weeks after injection, discoloration involved practically all vessels near the injection points in the trees left to stand until this time; it was widespread in most branches of the trees and had reached the soil level in six of seven trees examined in detail.



Discoloration developed slowly in side branches that came off the injected axis below the injection point in these trees. Only one of the branches that came off the injected axis below the injection point developed any discoloration in 30 trees felled and defoliated within a few hours or up to 1 day after injection. There was extensive discoloration in some of the lower side branches in trees felled and defoliated 5 days after injection. Of the branches below the injection point, those nearest the injection point developed discoloration first. The extent of discoloration in some of these large branches was as great as in those above the injection point 5 days after injection. It extended only a few feet into many at this time, however. Two weeks after injection there were still numerous lower lateral branches free from discoloration.

#### INJECTION OF SPORE SUSPENSION INTO TERMINAL SHOOT GROWTH OF CURRENT SEASON

Spores of *Ceratostomella ulmi* were injected into the terminal shoot growth of the current season at the top of trees 10 to 15 feet in height. The injections were made by excising these shoots 2 to 4 inches from their ends after bending them down and submerging the parts in a concentrated spore suspension in a pail. The cut ends were held under the suspension for 1 hour. The excised tips were discarded. The trees were then cut and defoliated and left lying in the shade of a dense thicket for a month before inspection for discoloration was made.

TABLE 10.—Distribution of *Ceratostomella ulmi* below points at which spore suspension was injected<sup>1</sup> for 1 hour into apical stem growth of the current season

Tree No.	Shoot No.	Maximum distribution of spores below injection point determined by—		Terminal stem growth of the current season	
		Vascular discoloration induced	Isolation of <i>C. ulmi</i> <sup>2</sup>	Total length remaining after injection	Length not invaded
		Inches	Inches	Inches	Inches
51.....	1	3.5	3.5	12.0	8.5
	2	6.0	6.0	12.5	6.5
	3	0	0	4.0	4.0
52.....	1	6.0	6.0	17.0	15.0
	2	5.0	3.0	18.0	13.0
	3	3.0	3.0	19.0	16.0
53.....	4	6.0	6.0	19.0	13.0
	1	3.0	3.0	20.0	17.0
	2	3.0	3.0	19.0	16.0
54.....	3	6.0	6.0	22.0	16.0
	4	3.0	3.0	19.0	16.0
	5	3.0	3.0	21.0	18.0
55.....	1	4.5	3.0	8.0	3.5
	2	6.0	5.3	18.0	12.7
	3	2.0	2.0	13.5	11.5
56.....	4	2.0	.5	14.5	12.5
	1	2.5	2.5	6.0	3.5
	2	6.0	4.0	17.0	11.0
57.....	3	5.0	1.5	17.5	12.5
	4	4.0	3.0	14.8	10.8

<sup>1</sup> American elms injected Aug. 22, 1938, by excision under a suspension of *C. ulmi* spores. The point at which the stems were cut was 2 to 4 inches from their ends.

<sup>2</sup> 3 weeks after injection every inch of stem bearing vascular discoloration was cultured in trees 51, 52, and 53. The injected shoots were also cultured at 6, 12, 18, and 24 inches below the point of injection.

<sup>3</sup> Isolations from injected shoots of trees 54 and 55 were not made until February 1939.

Discoloration did not extend more than a few inches below such injection points in terminal stem growth of the current season, nor did it reach older wood in any of the terminal shoots injected. The diameters of the portions of the shoots invaded by *Ceratostomella ulmi* ranged from one-sixteenth to one-eighth of an inch. The organism injected was isolated in most cases to the limits of the discoloration recorded in early trials but not below the limits of discoloration (table 10). The downward distribution of the fungus in current season's stem growth was thus in striking contrast to that found for spores injected into the top of the bole or major branches of the elm.<sup>7</sup>

#### DISTRIBUTION OF SPORES OF CERATOSTOMELLA ULMI IN TOP-INOCULATED ELMS

Under natural conditions, spores of *Ceratostomella ulmi* probably are deposited in injured tissues rather than injected into the sap stream. To gain some concept of the rapidity of invasion of the vascular system under more natural conditions, a series of trees were top-inoculated and the results compared with those from trees that had been top-injected.

Inoculations were made into the tops of 27- to 49.5-foot trees July 16, 1937. The inoculations were made into the leader branch or

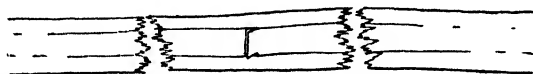


FIGURE 9.—Diagrammatic sketch of initial vascular invasion by *Ceratostomella ulmi* from a chisel-cut inoculation on a leafy elm branch. Streaks of discoloration from which *C. ulmi* was isolated appeared in line with the edges of the chisel cut 1 week after inoculation in July. The streaks were caused by invasion of the fungus in the functional vessels nearest the inoculated tissue.

branches within 5 to 15 feet of their ends. The diameters of the branches at the inoculation points ranged from 0.5 to 1.75 inches. The inoculated terminal branches or the entire trees were cut off 7, 19, and 116 days respectively, after inoculation. The branches removed after 7 and 19 days, respectively, were stripped of leaves, left in the shade for 2 or more weeks, and then examined for spore distribution by the discoloration procedure. The others were examined at once.

Seven days after inoculation of five branches treated in this manner, four showed no discoloration above or below the injury. In the fifth branch, streaks of discoloration in line with the edges of the chisel injury extended 14 inches above the injury and 10 inches below (fig. 9). The streaks were discolored vessels of the current season's growth sheath. There was but one streak on either side of the injury. The streaks in each instance involved several vessels (fig. 4, B).

The extent of vascular invasion 19 days after inoculation in 12 branches examined at this period is shown in table 11. Typical wilt symptoms of Dutch elm disease were shown by only 4 of these 12 branches at this time. Vascular discoloration extended into terminal shoot growth of the current season in all 4. Vascular discoloration was extensive above the inoculation points in 7 of the branches examined; it was limited in the other 5, however, to within 1 inch of the

<sup>7</sup> The results of Radulescu's (29) leaf-injection experiments, described in the literature review of this paper, are in decided contrast to the results obtained in these experiments.

inoculation points. Below the inoculation points, vascular discoloration extended on an average to roughly but two-thirds of the distance to which it extended above the inoculation points. Representative areas of the discolored tissue were cultured at the time of observation and *Ceratostomella ulmi* was consistently recovered from them.

TABLE 11.—Distribution of *Ceratostomella ulmi* from inoculation points <sup>1</sup> in terminal branches of American elms 19 days after inoculation

Tree No.	Branch		Tree height	Wilt symptoms <sup>2</sup> Aug. 4	Stem below inoculation point			Stem above inoculation point			
	No.	Diam-eter at inoculation point			Total length	Lower limit of vessel discoloration	Vessels discolored <sup>3</sup>	Total length	Terminus of discoloration		
									Height above inoculation point	Distance below end of branch	Stem age
		<i>Inches</i>	<i>Feet</i>		<i>Feet</i>	<i>Feet</i>	<i>Number</i>	<i>Feet</i>	<i>Feet</i>	<i>Inches</i>	<i>Years</i>
12.---	3	0.50	30.4	—	22.0	0	0	8.1	0	84	9
	4	.50		—	20.0	.25	1	6.5	.6	96	9
	5	.75		—	23.0	.9	1	7.4	.7	70	7
	6	.63		+	22.0	5 6.0	—	7.6	7.5	3	1
22.---	1	1.3	49.5	+	34.1	5.0	—	11.1	10.2	10	1
	2	1.75		+	34.5	8.7	6	15.0	12.5	30	5
	3	1.5		+	34.3	8.7	—	15.0	14.8	4	1
	4	1.25		+	33.7	4.8	—	10.6	10.2	4	1
23.---	1	.75	40.2	—	30.0	0	0	10.2	.1	121	9
	2	1.20		—	26.5	3.8	3	13.5	1.0	150	10
25.---	2	1.0	43.0	—	31.7	1.2	2	8.9	5.8	37	4
	3	1.1		—	27.	5 8.7	7	8.5	6.0	30	4

<sup>1</sup> Spores of *C. ulmi* were placed on the surfaces of chisel cuts made in the wood, July 16, 1937.

<sup>2</sup> Plus sign (+) indicates presence; minus sign (—), absence of symptoms.

<sup>3</sup> *C. ulmi* was recovered by isolation at representative points from the vascular discoloration recorded in this table.

<sup>4</sup> The discolored vessels were in line with the edges of the chisel inoculation cuts as shown in figure 9.

<sup>5</sup> Vascular discoloration extended into the bole below the point at which this branch was cut from the tree. No observations were made below this point at this time.

Approximately 4 months after inoculation, all the remaining inoculated trees were felled, examined for vascular discoloration, and destroyed. Data for seven trees in which the extent of invasion above and below the inoculation point was traced in detail are shown in table 12. Foliar symptoms of disease had appeared only on the three trees in which vascular discoloration had reached the terminal shoot growth of the current season and in which nearly all vessels of the outermost ring in the upper half of the bole were discolored. Vascular discoloration was present in the stump, however, in only one of these three trees. In the other four trees it was limited to a few vessels in line with the edges of the chisel-cut inoculation injury.

Eleven of the 25 trees top-inoculated displayed symptoms of disease by September 15, 1937. Three of these are shown in table 12. Vascular invasion had reached the stump in only 4 of these trees by November 9, 1937. The 13 other trees showed no symptoms at any time, yet in 3 of these trees vascular discoloration extended into the stump by November 9. Data for one of these trees (tree 25) are shown in the table. *Ceratostomella ulmi* was found associated with the vascular discoloration recorded in these trees at practically all representative points cultured. It is apparent from these experiments that although the fungus may be distributed from top to bottom and throughout most branches of a tree within a few hours after injection, its distribution subsequent to its establishment in an injury is not predictable.

TABLE 12.—Distribution of *Ceratostomella ulmi* from inoculation points<sup>1</sup> in terminal branches of American elms, 116 days after inoculation

Tree No.	Branch		Tree height	Wilt symptoms <sup>2</sup>		Stem below inoculation point			Stem above inoculation point			
	No.	Diam-eter at inoculation point		Aug. 4	Sept. 15	Total length	Lower limit of vessel dis-colored	Vessels dis-colored <sup>3</sup>	Total length	Terminus of discoloration		
										Height above inoculation point	Distance from end of branch	Stem age
		<i>Inches</i>	<i>Feet</i>			<i>Feet</i>	<i>Feet</i>	<i>Number</i>	<i>Feet</i>	<i>Feet</i>	<i>Inches</i>	<i>Years</i>
6	1	0.80	48.0	—	+	34	26.0	∞	14.0	13.7	5	1
19	1	1.25	37.3	+	+	29	22.0	∞	8.3	8.0	5	1
20	1	1.25	42.0	—	—	28	12.0	54	14.0	7.0	24	4
23	1	1.50	40.2	—	—	27	8.0	54	13.0	3.0	120	9
24	1	.75	27.0	+	+	19	19.0	∞	8.0	7.8	2	1
25	1	1.50	43.0	—	—	30	1.5	53	13.0	5.0	96	10
25	3	1.1	43.0	—	—	27	27.0	56	68.5	6.0	30	4

<sup>1</sup> Spores of *C. ulmi* were placed on the surface of chisel cuts made in the wood July 16, 1937.<sup>2</sup> + indicates presence, — absence of symptoms.<sup>3</sup> *C. ulmi* was recovered by isolation at representative points from the vascular discoloration recorded in this table.<sup>4</sup> Nearly all vessels of the outer ring were discolored in the upper part of the bole in these trees.<sup>5</sup> The discolored vessels were in line with the edges of the chisel inoculation cuts as shown in figure 9.<sup>6</sup> This branch was excised August 4, 1937.

### PRESENCE OF SPORES OF CERATOSTOMELLA ULMI IN SAP DISPLACED FROM ELMS AFFECTED BY DUTCH ELM DISEASE

The rapid progress of *Ceratostomella ulmi* in the vessels of trees into which no spores were injected is evidence that the fungus produces spores or free drifting mycelial fragments in the vessels to which it gains access by tissue penetration in the vicinity of the injuries involved in the inoculation procedure. Spores have been seen in histological preparations of the discolored vessels of stems from naturally diseased and from inoculated trees (4). Verification of the presence of spores in the vessels of stems of diseased trees at various times during the leafy season of 1936 was obtained experimentally by the following procedure.

TABLE 13.—Presence of *Ceratostomella ulmi* spores in sap displaced from American elms affected by the Dutch elm disease, 1936

Date	Tree designation	Height at which sap was displaced	Yeast-like spores in centrifuged sap	<i>C. ulmi</i> colonies identified in each plate <sup>1</sup>	Date	Tree designation	Height at which sap was displaced	Yeast-like spores in centrifuged sap	<i>C. ulmi</i> colonies identified in each plate <sup>1</sup>
		<i>Feet</i>		<i>Number</i>			<i>Feet</i>		<i>Number</i>
June 15	9B	2-4	—	3, 3, 3	Aug. 17	K <sub>1</sub>	22-26	+	0, 0, 0, 0
Do.	9B	16-20	+	7, 14, 8	Do.	K <sub>3</sub>	36-40	+	0, 0, 0, 0
July 1	21B	2-4	+	—	Aug. 18	1071B	12-16	+	0, 0, 0, 0
Do.	21B	12-16	+	—	Do.	107B	12-16	+	3, 2, 4, 3
July 3	10B	4-7	+	0, 0, 0, 0	Do.	1072B	8-12	—	0, 0, 0, 0
Do.	10B	7-11	+	0, 0, 0, 0	Do.	K <sub>2</sub>	36-40	+	10, 23, 5, 9
Do.	10B	12-15	+	12, 3, 2, 0					

<sup>1</sup> 1 cc. of the optimum dilution of the sap sample was flooded over each of 3 or 4, 9-cm. Petri dishes containing solidified acid malt agar.<sup>2</sup> — signifies absence; +, presence of yeastlike spores of *C. ulmi*.

Sap was displaced from 2- to 4-foot sections of the stem of diseased trees at various periods of the year. Naturally diseased (K) and "inoculated" trees (B) were used. The work was done only on those trees that displayed characteristic foliar symptoms of disease during the leafy season of 1936. All organisms contained in the displaced sap were concentrated into a 0.2-cc. volume by centrifuging. Yeast-like spores (fig. 3), typical of those produced by *Ceratostomella ulmi* in liquid culture, were seen in most samples (table 13). The 0.2-cc.

concentrates were resuspended as described for this technique earlier, and the suspensions were cultured. Colonies of *C. ulmi* were identified in cultures of the sap displaced from four of nine trees studied.

#### DISTRIBUTION OF DYES SUBSEQUENT TO INJECTION INTO ELMS AT VARIOUS SEASONS

Dyes were injected into elms at various seasons of the year. Their distribution served as an index of the movement of the sap stream from injection points to other parts of the tree. The pan and chisel-cut injection procedure was used. The injection periods ranged from 1 to 84 hours. The trees were felled at the end of the injection period, at which time the locus and extent of dye distribution in trunk and crown of the injected trees were traced by inspection for dye in cross sections of the stem. Acid fuchsin and light green were the dyes used. Trees in the same stands and similar to those used in the spore-distribution experiments were employed. A record of the limits of distribution of these dyes in trees base-injected at various seasons of the year appears in table 14.

The dyes were observed in the lumina of many of the tracheae of small diameter of all growth sheaths severed by the injection chisel. They also appeared in the walls of contiguous cells. The dyes rose for several feet in this system of vessels in trees injected during the leafy season. The vessels of large diameter of the new ring, however, were the channels whereby dye was distributed throughout the crown in the leafy season. The dyes were not observed in tracheae of large diameter of any ring in those trees injected during the spring flowering period or during the dormant season.

TABLE 14.—Distribution of water-soluble dyes injected stump high into American elms, 1936-37

Tree No.	Date	Seasonal condition of tree	Injection time	Tree dimensions		Maximum height reached by dye
				Diameter breast high	Height <sup>1</sup>	
				Inches	Feet	Feet
1	1936 Apr. 13	Flowering, no leaves	72	3	20	5.2
2	do	do	72	4	29	7.8
3	1937 Apr. 26	do	3	5	59	4.2
4	do	do	3	7	59	3.3
45	May 11	New leaves expanding	2	6	40	22.0
47	do	do	2	4	35	20.0
48	do	do	2	4	35	25.0
5	July 19	Full leaf	18	9	55	55.0
6	do	do	18	9	55	53.0
7	1936 Sept. 10	do	3	4	31	30.0
8	do	do	3	4	27	26.3
9	Sept. 24	do	3	3	25	24.6
10	do	do	18	5	35	35.0
11	Nov. 3	Leafless, dormant	3	5	34	6.0
12	do	do	3	4	22	6.3
13	do	do	3	4	30	4.2
14	Dec. 3	do	84	8	48	21.0
15	do	do	84	10	57	21.0

<sup>1</sup> Measured from the point of injection.

<sup>2</sup> Chemically girdled trees in which the cambium was killed, during the preceding dormant period, over a 3- to 10-foot length of trunk at the base of the tree.

At the season of the year when the new shoots were rapidly elongating and the new leaves expanding, and at a time when the vessels of the new ring were not yet mature at the base of the trees, the dye rose 10 to 15 feet in the small tracheae of the growth sheaths penetrated by the chisel used in making the injections. It was distributed farthest, however, in the new tracheae of large diameter of the new ring, which were present in the upper half of the bole and crown at this time although not yet mature in the lower part of the bole. When trees were injected during the leafy season, the dyes were observed in the walls of the tracheae of the outer ring of large vessels and in contiguous cells in the crown. These vessels were colored by the dyes throughout the crowns of the trees injected during the leafy season to the heights indicated in the table. When injection at this season was continued for 18 hours all leaves were stained red by the acid fuchsin.

Dyes were injected into the girdled area of leafy trees that had been banded with copper sulfate during the preceding dormant season, by a procedure similar to that described by Bedard (6), Lantz,<sup>8</sup> and Liming.<sup>9</sup> The cambium in such trees had been killed over a distance of 3 to 10 feet above the girdle all around the trunk. The dyes after injection into these trees overnight were observed in the lumina of many tracheae of small diameter scattered through the wood of all the growth sheaths penetrated by the injection chisel up to 35 feet above the injection point. Although such dye-conducting vessels were generally scattered at random through all sap rings penetrated by the injection chisel (fig. 4, C), there was a pronounced tendency toward dye conduction in rings of vessels. These rings of color seemed to coincide with the rings of large spring vessels. Close inspection revealed, however, that dye was in the lumina of only the small vessels of the late summer wood contiguous to these rings. There was no convincing evidence that dye was distributed in the vessels of any of the rings of large tracheae formed prior to the season of injection. Dye was widely distributed in the new ring of large spring vessels formed above the girdled area. It was observed in this system of vessels even in the small branchlets at the top of the two trees studied.

Dyes were injected into the top of the boles of a few trees during the leafy and dormant seasons. The path of distribution was the same as for base-injected trees. The distance to which the dyes descended below the injection point varied. After 72 hours of injection in November in one tree at 30 feet above the ground, light green was detected in the tissue around the outer ring of large vessels at 25 and 27 feet below the injection point. Three hours after injection in two leafy trees at 20 feet above the stump October 1, acid fuchsin was seen in a few vessels 6 inches above the stump in one of them. In the other it descended only 12 feet 10 inches below the injection point. It descended in the outer ring of vessels in these trees to branches at various distances below the injection point, then moved out toward the leaves in these branches, in some cases reaching the leaves. Light green followed a similar course in the trees injected after leaf fall, but did not reach the distal portion of the lateral branches.

<sup>8</sup> LANTZ, A. E. AN EFFICIENT METHOD FOR INTRODUCING LIQUID CHEMICALS INTO LIVING TREES. U. S. Dept. Agr., Bur. Ent. and Plant Quar. E 434, 4 pp., illus. 1938. [Mimeographed.]

<sup>9</sup> See footnote 6.

## RATE OF DISTRIBUTION OF HIGHLY COLORED SUSPENSIDS AND STAINED YEAST CELLS INJECTED INTO ELMS

Highly colored suspensoids and stained yeast cells were injected into elms (*Ulmus americana* and *U. fulva*) at various seasons of the year. The pan and chisel-cut injection procedure was used. The distribution of stained spores or suspensoids was observed in situ in the new ring of vessels at the season of the year when little wood had as yet been laid down over the new vessel ring. In such cases, the bark was removed immediately before injection. The highly colored particles injected moved through the vessels of the new ring for varying distances and produced in these vessels the same spotted or discontinuous streak pattern that the various elm wilt fungi generally induce. These suspensoids during the months of June and July commonly ascended the first 5 feet in 8 to 15 seconds and reached their maximum upward distribution of 8 to 20 feet in 2 to 10 minutes. They descended from injection points in the bole at the base of the crown with like speed. Two minutes after injection, June 24, 1937, india ink had descended to 14.5, 18.3, and 19 feet in three trials from injections made 21 feet above the stump. It descended 36 feet in 6 minutes from an injection made June 25, 1937, 38 feet above the stump. All this distribution occurred in the outer or new ring of vessels. The carbon particles of india ink, suitably diluted, Turnbull's blue, iron oxychloride, and beer yeast cells stained with Heidenhain's iron-alum haematoxylin were not distributed in any of the large spring-formed vessels of inner rings but were distributed to a distance of less than 16 inches in the vessels of the summer wood of all growth sheaths injected. During the nonleafy period of the year, distribution of these suspensoids was limited to the vessels of the summer wood but did not exceed 16 inches above or below injection points made in the boles of trees 6 to 12 inches in diameter breast high.

## DISCUSSION

## RELATION OF DRIFTING SPORES TO EXTENSIVE RAPID FUNGUS INVASION OF THE TRACHEAL SYSTEM IN THE ELM

The experimental evidence presented demonstrates that rapid and extensive spread of *Ceratostomella ulmi* in the American elm is due to the distribution of its spores, drifting in the sap stream. Limited evidence presented indicates that the two other fungi known to induce vascular wilt diseases of the elm, namely, *Verticillium dahliae* and *Dothiorella* (*Cephalosporium*) *ulmi* are distributed in the same way in the elm. Experiments<sup>10</sup> with living cells of the beer yeast *Saccharomyces cerevisiae* Hansen have demonstrated that these cells may be distributed with like speed and essentially as far in the vascular system of the American and the slippery or red elm. From this it seems probable that other organisms would be distributed in the same manner, if their spores were produced in the functional vessels in question.

Spores of *Ceratostomella ulmi* have been seen and identified in sap collected from all levels of the boles of injected trees, up to 35 feet in height, within a few hours after injection at one point. They have

<sup>10</sup> See footnote 4.

been observed in sap taken from the boles of diseased trees months after initial infection. They have been seen in histological sections in vessels of small branches of the crowns and of the boles of inoculated and naturally diseased trees (4).

The presence of any of these organisms in functional vessels has been found in these studies to be consistently associated with discoloration of the vessels so occupied. This discoloration has been produced by all three fungi to the limits of their distribution but not elsewhere. It has been traced throughout the crown and bole of trees that have been felled, sectioned into 3- or 4-foot lengths, and defoliated within a few hours after spore injection, and in most cases the fungus injected has been demonstrated to be present in the discolored tissues but not elsewhere.

Invasion of elm tissue by mycelial growth of *Ceratostomella ulmi* is exceedingly slow, comparatively. Within the several hours' time in which the spread of *C. ulmi* spores occurred in the vessels of certain trees injected with spore suspensions of *C. ulmi*, spores in uninjected portions of these suspensions held in Erlenmeyer flasks in the laboratory and on the ground in the vicinity of the injected trees were found to reproduce almost exclusively by yeastlike multiplication. Spores from suspensions that had been thickly sown on nutrient agar media and had been held under the same conditions also reproduced practically exclusively by yeastlike multiplication. The maximum distance to which *C. ulmi* can grow along the lumina of vessels under optimum conditions is not precisely known. Initial trials,<sup>11</sup> however, have demonstrated a growth of 4 cm. parallel to the vertical axis of stems when they were held in a moist chamber for 2 months. It is obvious, therefore, that distribution by growth to the limits found in these experiments would be impossible within the time indicated.

These findings provide a new and fundamental concept of the mechanics of invasion in these and probably other vascular wilt diseases.

#### VESSEL LENGTH IN RELATION TO SPORE DISTRIBUTION

The distribution of spores or suspensoids in the elm is conditioned by the length of the tracheae into which they are introduced. De Bary (5) concluded that "As regards the absolute size of vessels, there is nothing to oppose the view that their length may equal that of the whole plant or at least may be very great." Strasburger (34), and more recently Priestley and his associates (26, 27, 28) have presented experimental evidence that supports this view as to the length of tracheae, at least in ring-porous dicotyledons. That there should be vessels open from base to terminal branches in the crown of elms regardless of the height of the tree was, therefore, to be expected. Extensive rapid distribution of spores throughout American and slippery elms occurs only in the new or outermost ring of vessels. The distribution of spores within a few hours to days after injection was found in this work to be limited to a few inches from points of injection in leafy American elms prior to the maturation of these vessels. It was likewise limited in girdled areas of leafy American elms in which no new ring of vessels was formed.

<sup>11</sup> See footnote 4.



## ROLE OF SAP STREAM IN DISTRIBUTION OF SPORES

The role of the sap stream in the distribution of the wilt-inducing fungi studied is apparent from comparison of their spread from injection or inoculation points in dormant trees with that in leafy trees. When injected into dormant trees they were not spread more than a few inches in several weeks from points of introduction, whereas in the leafy season they were spread throughout the greater part of trunk and crown of small or large trees within a few hours. The speed of spore and water movement from injection points in leafy trees, judged from observation of dye and colored suspensoids in situ, is apparently a matter of several feet in a few seconds in either direction from injection points. Preston (25) reports initial rates of injections from dilute india ink of 1 m. per second in *Fraxinus americana* L. The distribution of spores of *Ceratostomella ulmi* above points of injection or inoculation in the elm is a function of the transpiration stream.

The reversal of the direction of this flow, as shown by the downward movement of dyes, suspensoids, and spores in these experiments, is similar to the reversal in the flow through the xylem reported more than two centuries ago by Hales (19) and in recent years by Arndt (1), Dixon (14), Dixon and Ball (15), and Yendo (42). The experiments of these investigators show that under the experimental conditions induced, tension created by the drawing off of water from transpiring leaves "determines a flow from any source wherever situated, and the continued transpiration from the leaves draws the supply through the plant along the channels of least resistance" (14, pp. 61-62). This loss of water by transpiration exceeds water intake, is cumulative, and induces "saturation deficits" in the tissue during the leafy seasons, according to Shull (32), and Smith, Dustman, and Shull (33). The downward translocation of liquids in the vascular system as in these experiments, as well as the initial rapid translocation above injection points, is thus a consequence of saturation deficits in the bole and roots of the elm, which would be relieved from any direction by a supply of free water, and, in Shull's words (32), "has nothing to do with the normal transpiration or translocation currents" in the plant. It would seem obvious, therefore, that this same rapid distribution of spores in either direction from points of injection in the bole of the elm would occur at any time during the leafy season, by night or day, in sunshine or rain. Experience has shown that this is essentially what happens.

## INJECTION OF SPORES IN NATURE

The injection of spores into functional vessels of the elm apriori might seem to be a highly artificial procedure. However, any agency that severed these vessels would bring about the injection during the leafy season of such micro-organisms as happened to be in suspension in free liquid at the point of vessel rupture. In nature it would seem that injections of this type might be made by bark beetles. Since there is ample evidence that certain of these beetles carry spores of *Ceratostomella ulmi* externally and internally (7, 16, 24, 32), these spores, as well as other micro-organisms, probably are present in cell sap released by the burrowing of these insects. At the moment a functional vessel is severed, some of this liquid and any spores suspended therein would be drawn into the vessel.

Streaks of discoloration that extend from bark beetle galleries have been reported on various ring-porous trees (20). From such streaks on elm, *Ceratostomella ulmi* has been isolated by a number of investigators. Streaks of this kind were repeatedly seen on elms felled by the writer in early June and examined within 10 days after felling. Usually they extended for a distance of several feet above and below the point of vessel rupture. These streaks consisted of individual vessels or small groups of vessels of the new ring, which, as cultural study demonstrated, had been invaded by various fungi and bacteria. These organisms gained access to the vessels at the point ruptured by galleries of *Scolytus* or *Hylurgopinus*. Such a distribution appears to have been effected by the movement of sap or rain water in either direction from the point of rupture in these vessels and not by growth of the organism involved. India ink injected into the bole of recently felled elms was distributed in the same manner several feet above and below the point of vessel rupture or injection.

The bark beetles are the only demonstrated means, except root grafting, of transmitting *Ceratostomella ulmi* from diseased to healthy trees in nature (12, 17, 23, 30). Injection of spores into functional vessels by these beetles probably occurs, although inoculation, i. e., the deposition of the fungus on the surface of injured tissue, from which it could grow into functional vessels, might be the more usual method.

#### DISTRIBUTION OF CERATOSTOMELLA ULMI FROM INOCULATION POINTS

The distribution of *Ceratostomella ulmi* from inoculation points occurs much more slowly than from injection points. Particularly is this true of distribution below inoculation points in the bole or crown of large trees. It results from several factors. (1) The fungus first has to gain access to functional vessels by mycelial growth from the inoculated tissue. (2) Spores must be produced in the invaded vessels. (3) The distribution of these spores below inoculation points probably depends either on reversals in the flow of sap in the invaded vessels or on precipitation of spores by gravity in invaded vessels wherein upward sap flow has been brought to a standstill by extensive tylose formation, which in most cases follows invasion by the fungus (8). Dr. A. L. Smith<sup>12</sup> found that some of the spores present in a spore suspension of *C. ulmi* fell through a still water column 5 feet in 30 minutes. (4) It would seem that invasion, production, release, and distribution of spores must occur in advance of occlusion of the areas of vessels occupied by tyloses.

#### VARIABILITY IN EXTENT OF VASCULAR INVASION IN ELMS FOLLOWING INOCULATION WITH THE WILT-INDUCING FUNGI

Finally it should be remembered that, although spores of the wilt-inducing fungi may be distributed throughout the greater part of the current season's growth sheath in even large trees within a few hours after such highly artificial injection procedures as are described in these experiments, invasion by these fungi under natural conditions is probably highly variable. This is suggested by the variable results that follow inoculations with these fungi. General and rapid invasion has practically always resulted, in the writer's experience, from chisel-cut injections with *Ceratostomella ulmi* spores, made during

<sup>12</sup> See footnote 4.

the month of June into the bases of American and slippery elms. No such invasion was found in dormant trees in these experiments. After the July top-inoculation experiments described in this paper, invasion of the vascular system of American elms was erratic, in most cases limited to the crown, and generally unpredictable. Smucker<sup>13</sup> found invasion to be limited to within a few centimeters of inoculations made with *C. ulmi* in the summer wood of crotches in the crowns of 4-foot trees treated in late July. Moreover, discoloration to the limits indicated was found in only 21 of the 50 cases studied. Invasion was likewise localized in 31 of 35 trees wherein the inoculation injury severed the functional ring of vessels in crotches during July. Invasion was also limited to within a few centimeters of similar inoculations in 31 of 40 trees wherein inoculation was made at the same time in the upper part of the trunks. Examination was made in the latter two groups 1 month after inoculation.

Variable distribution of *Ceratostomella ulmi* in elms in nature is evident from the reports of Ahrens, Shuttleworth, and McMaster,<sup>13</sup> and of True and Slowata (35). The first-named writers reported on 185 trees that were found to have recurrent symptoms of Dutch elm disease during the season following that in which the earliest ring vessels discolored, by the fungus were produced. They stated that vascular invasion during the season of initial infection was confined to one-fourth of the crown in 40 of these cases and that it was general throughout the entire tree in but 108 cases. Their study was based on discoloration in the penultimate ring of vessels from which *C. ulmi* was isolated. True and Slowata, reporting on a study of symptoms of Dutch elm disease in natural infections during the leafy season, stated that "infections unaccompanied by symptoms were mostly localized" in the large elms they studied.

Infections by *Cephalosporium* in most cases remain confined to a "limited portion only of the current season's growth" in infected twigs during the season of infection, according to Creager (13). Rapid invasion of the entire tree generally followed from the inoculations made at the base of the trunks of small trees by Goss and Frink (18).

*Verticillium* infections generally are believed to occur through the soil. Wollenweber (40), however, reported invasion of the vascular system as confined to within a few inches of points of inoculation in leafy trees in the autumn.

## SUMMARY

### METHODS

Spore suspensions of *Ceratostomella ulmi* were injected in relatively large volumes (0.1 to 89 liters) into the bases or tops of small and large elms during all seasons of the year. Several hours to 5 weeks after injection, determination of the limits and loci of distribution of the spores in these trees was made by one of two procedures. (1) Sap was displaced from representative 3- or 4-foot lengths into which the trees were cut immediately after completion of the injection period; the sap samples were examined for *C. ulmi* spores after concentration in a centrifuge; the spores observed were subsequently identified from the colony character and fructifications to which they gave rise when

<sup>13</sup> See footnote 4.

isolated on nutrient agars. Or (2) the trees were felled immediately at the close of the injection period, cut into 3- to 20-foot lengths at once, and then defoliated; the discoloration of the vessels through which spores had been distributed was traced several weeks after the injection; *C. ulmi* was isolated from the discolored vessels at representative points.

Small quantities of spores were also introduced in suspension through chisel-cut injections or were applied without free water to the surface of chisel-cut injuries at various points in the bole or crown of American elms up to 49 feet in height. Terminal shoots were injected by excision under spore suspension. Distribution of the fungus applied in these procedures was traced from the vascular discoloration induced and was confirmed by isolation of the organism.

#### RELATION OF SEASON TO DISTRIBUTION OF CERATOSTOMELLA ULMI IN THE ELM

Distribution of the fungus in the tree was profoundly influenced by (1) the season at which injection was made, (2) the locus of injection, and (3) the method of introduction into the tree.

During the early half of the leafy season *Ceratostomella ulmi*, injected in spore form in large volumes of suspension stump high, was recovered to within a few inches to feet of the terminal growth in small and large elms that had been felled and cut into 3- to 20-foot sections at the close of injection periods of 20 minutes to 48 hours. The average distribution was 10.7 feet in 9 small trees that averaged 12.9 feet in height, and 44.3 feet in 23 tall trees that averaged 49.6 feet in height. *Verticillium dahliae* and *Dothiorella* (*Cephalosporium*) *ulmi* were recovered within 1 to 11 feet of the tops of six 23- to 44-foot American elms 6 and 7 days, respectively, after injection of a few cubic centimeters of spore suspension at stump height. Injected in large volumes of spore suspension 40 or 41 feet above the stump at points where the stem was 4.5 or more inches in diameter, *C. ulmi* was recovered at stump height in trees felled at the close of 15- or 20-minute injection periods.

During the latter half of the leafy season, *Ceratostomella ulmi*, injected stump high in large volumes of spore suspension, could not be recovered at more than, roughly, one-half the height of twenty-five 13- to 60-foot trees, felled at the close of injection periods of 1 to 4.2 hours. Average maximum height at which the vessels were discolored by the spores was 10.8 feet in these trees, which averaged 29.7 feet in height. Injected 21 to 29 feet above the stump at this season in large volumes of spore suspension at points where the stem was 2 to 6 inches in diameter, *C. ulmi* was recovered at stump height in one of nine trees felled 17 hours to 5 days after inception of injection. The average maximum distance below the point of injection at which *C. ulmi* was recovered, however, was 19.3 feet in those trees wherein injection was made at an average height of 24.9 feet above the stump.

Spores of *Ceratostomella ulmi* were identified in sap displaced from various points in the boles or crowns of naturally diseased and inoculated trees that showed wilt symptoms in the months of June, July, and September, 1936.

During the nonleafy season, *Ceratostomella ulmi* could not be recovered at more than 24 inches above injection points in 12 trees

injected with large volumes of spore suspension and felled 1 hour to 28 days after the inception of injection. *C. ulmi* could not be recovered at more than 12 feet below similar injections made 13 to 24 feet above the stump in 10 trees injected 1 to many hours and felled 1 hour to 14 days after injection at this season.

RELATION OF METHOD AND LOCUS OF INTRODUCTION INTO THE TREE TO  
SUBSEQUENT DISTRIBUTION OF *CERATOSTOMELLA ULMI* IN THE ELM

Injected into terminal shoots for 1 hour, *Ceratostomella ulmi* could not be recovered at more than 6 inches below the point of injection in trees felled at the close of the injection period. Average distribution was 4 inches in 20 trials in August.

Injected into leader branches at points where the diameter ranged from three-eighths to five-eighths of an inch, *Ceratostomella ulmi* was recovered at 2 to 16 feet below injection points in trees felled 24 hours after injection in July. The average distribution below the injection point in 10 trials was 7 feet. The average height of injection was 16 feet.

Distribution of *Ceratostomella ulmi* from inoculation points was decidedly variable and took place more slowly than from injection points. One week after inoculation in July into top branches 0.5 of an inch to 1.75 inches in diameter, maximum distribution from inoculation points did not exceed 14 inches; 19 days after inoculation, maximum distribution was 14.8 feet; minimum, none; approximately 4 months after inoculation in 25 trees up to 49 feet in height, distribution varied from 1.5 feet from inoculation points to the entire branching structure and bole of the trees and was limited to a few vessels or was generally present in all large tracheae of the new annual ring in the crown structure. From comparable points at which a few drops of spore suspension were injected through chisel cuts in the nonleafy season, distribution at the end of 6 weeks did not exceed 19 inches above or below, and averaged 1.4 inches above, 5.4 inches below the cuts in 12 trees approximately 40 feet in height.

LOCUS AND RATE OF DISTRIBUTION OF SPORES AND OTHER COLLOIDS IN THE ELM

The tracheal system was the locus of spore distribution. Spores and suspensoids were distributed by the sap stream in the small vessels of the wood and in the large vessels of the outer annual ring. They were distributed through the smaller vessels of the wood of all growth sheaths severed by the injection chisel. The distribution in these vessels was never found to be greater than 18 inches above or below injections made at any season in trees felled within 24 hours after inception of injection. Distribution of spores beyond 18 inches was almost exclusively through the large vessels in the outermost or new ring.

Highly colored suspensoids and stained yeast cells were observed to move in the large vessels of the outer annual ring up and down from injection points in large, standing, leafy trees at relatively fast rates. These suspensoids usually ascended the first 5 feet in 8 to 15 seconds, reached a maximum distribution of 20 feet in less than 10 minutes, descended with like speed, and reached a maximum distribution of 36 feet in 6 minutes.

## CONCLUSION

It may be concluded that the rapid invasion during the leafy season of the bole and crown of the American elm by the fungi herein discussed is due to the distribution of the spores of these fungi in the large vessels of the new annual ring by the sap stream.

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# A HISTOLOGICAL STUDY OF SNAP BEAN TISSUES AFFECTED WITH BLACK ROOT<sup>1</sup>

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## INTRODUCTION

In previous papers (5, 6)<sup>3</sup> the writer described the symptoms and etiology of black root, a new disease of snap beans (*Phaseolus vulgaris* L.). Since vascular discoloration is such a prominent symptom of black root, it appeared that a further study of certain histological details of diseased plant parts would contribute to a better understanding of the effects of the disease on the physiology and vitality of the entire plant. The results of such a study form the basis of this paper.

Several investigators have reported the results of anatomical and histological studies on various plants affected with virus diseases (1, 2, 4, 9). Others have reported disease symptoms on various plants, which, if studied histologically, would doubtless yield similar results (7, 10, 11). The remarkable degree of concurrence of opinion and observation on numerous critical phenomena found in viroseed tissues supposedly caused by unrelated viruses, attest to the interest in work of this kind and suggest the ultimate fruitfulness of this approach to a better understanding of the interrelationship of plant and virus physiology; even, perhaps, to interrelationships of viruses themselves.

## MATERIALS AND METHODS

Several varieties of snap beans, exhibiting various intensities of symptoms, in addition to several strains of beans originating from hybrid progenies, were used.

Both fresh and fixed materials were studied, the latter being killed in either Crafts fixative or formal-acetic alcohol, embedded in paraffin, sectioned and stained. Fresh material was used exclusively in free-hand sections, while the embedded material was cut 10 $\mu$  to 15 $\mu$  in thickness with a rotary microtome.

Various stains were used, but of these Pianese IIIb, alcoholic safranine counterstained with light green in clove oil, and Heidenhain's iron-alum haematoxylin proved best for the general purposes of this work. Certain details relating to suberin and cellulose deposition were confirmed microchemically by appropriate reagents. However, several points could best be determined from unstained sections which afforded an excellent check, particularly on the patterns of pathological cells.

With Pianese IIIb, healthy tissues stained various shades of magenta, except the middle lamellae of vessels, which stained green. The contents of diseased phloem and cambium tissues stained from light

<sup>1</sup> Received for publication July 26, 1940. Paper No. 76, Journal Series, Georgia Agricultural Experiment Station.

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<sup>3</sup> Italic numbers in parentheses refer to Literature Cited, p. 690.

elm green to dull blackish green (8), depending on the severity of involvement and, apparently, on the length of time the elements had been undergoing degenerative changes. The contents of those elements that showed the earliest symptoms of disease usually stained the deepest green, while the walls always stained magenta. Likewise gum,<sup>4</sup> when present in vessels, stained green.

With safranin-light green, the cell walls and contents of healthy phloem and cambium tissues stained green, with the exception of the nuclei and slime bodies which stained red. Lignified walls of the xylem tissues likewise stained red. The cell walls and contents of diseased tissues, both phloem and cambium, stained from Pompeian red to dark hyssop violet. In fact, the degree and intensity of red stain in the walls of these tissues afforded a true index to pathological involvement, even before marked degenerative changes in the cell contents were evident.

With Heidenhain's haematoxylin, healthy cells stained lightly in various shades of blue to purple, while diseased tissues stained various shades of olive to almost black. Its use greatly facilitated the photographic work.

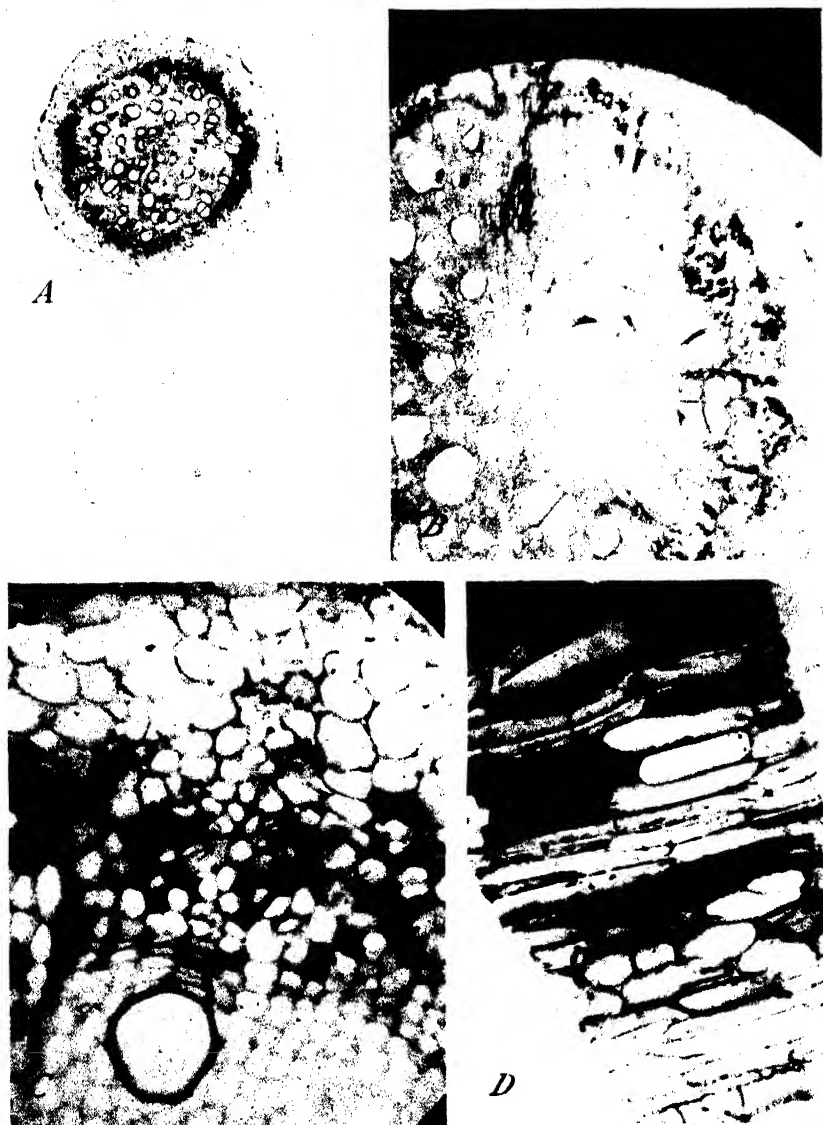
### HISTOLOGY OF THE ROOT

As stated in an earlier paper (6), the taproot of diseased plants never escapes infection. Exteriously, infected roots exhibit a generalized dark to almost black color, depending on the severity of infection. It has been assumed that the discoloration is due to certain chemical changes attendant on the death of the protoplasm, perhaps oxidation, and no additional explanation has come to light from this investigation.

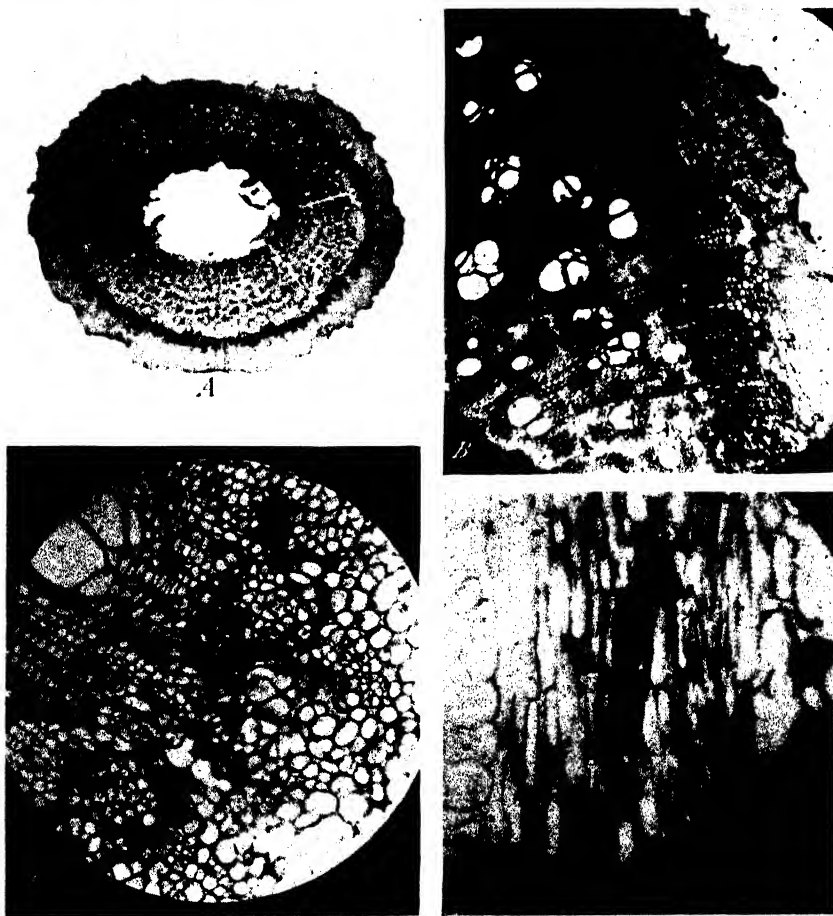
#### PHLOEM

In transverse and longitudinal sections of the diseased root (pl. 1), the visibly affected cells are seen to be confined almost exclusively to the phloem and cambium, although in certain cases discoloration is to be noted in the ray tissue and in the parenchyma surrounding the outermost layer of vessels. Under low magnifications all the tissues of a given region seem to be affected, but when higher magnifications are used (pl. 1, *B, C, D*), numerous areas showing no symptoms of disease are apparent. Conditions are readily demonstrable in given areas, particularly of the phloem in which the individual cells show symptoms ranging from isolated areas in portions of certain cell walls, that stain red, to those in which the cytoplasm and nucleus have undergone complete disorganization, now appearing as amorphous or gumlike inclusions that stain intensely red throughout (pl. 1, *B, C*). The young phloem fibers show symptoms of involvement, particularly during the early stages of differentiation. Judging from the reactions of the cell walls to the several stains employed and to Sudan III, IV, and Sudan black, it is fairly obvious that the cellulose walls of the phloem (including the fibers) have become either in part or completely covered by a deposit of suberin. Such a situation was demonstrated microchemically by Hill and Orton (4) in the course of histological studies on blue stem of potatoes. That the suberin is in some way correlated with protoplasmic disorganization is attested by the fact

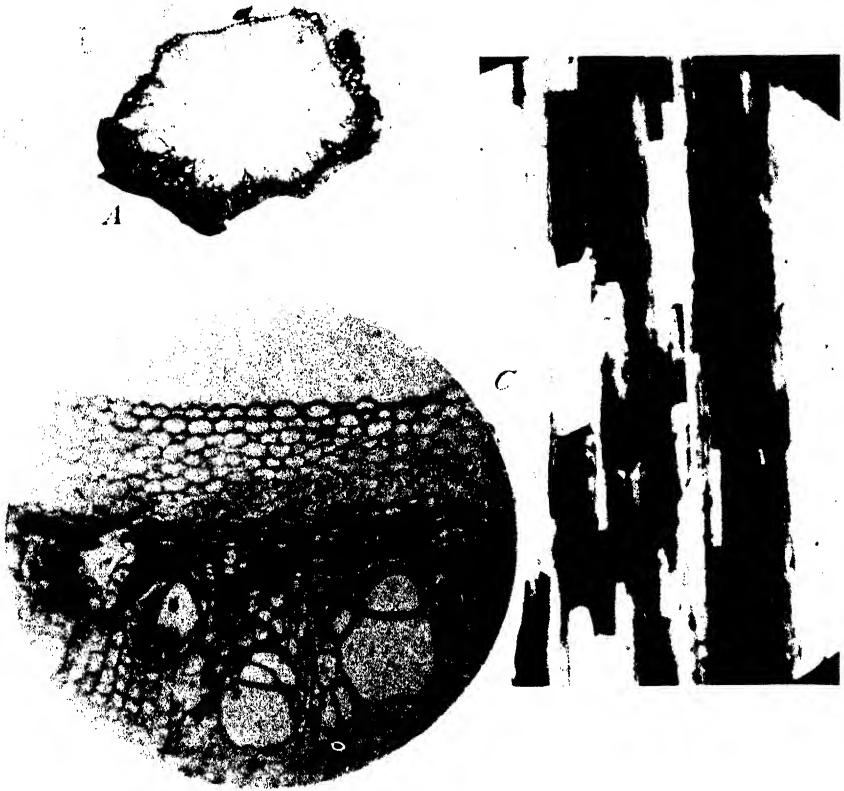
<sup>4</sup> The term "gum" refers to the physical appearance of the substance, rather than to its chemical composition.



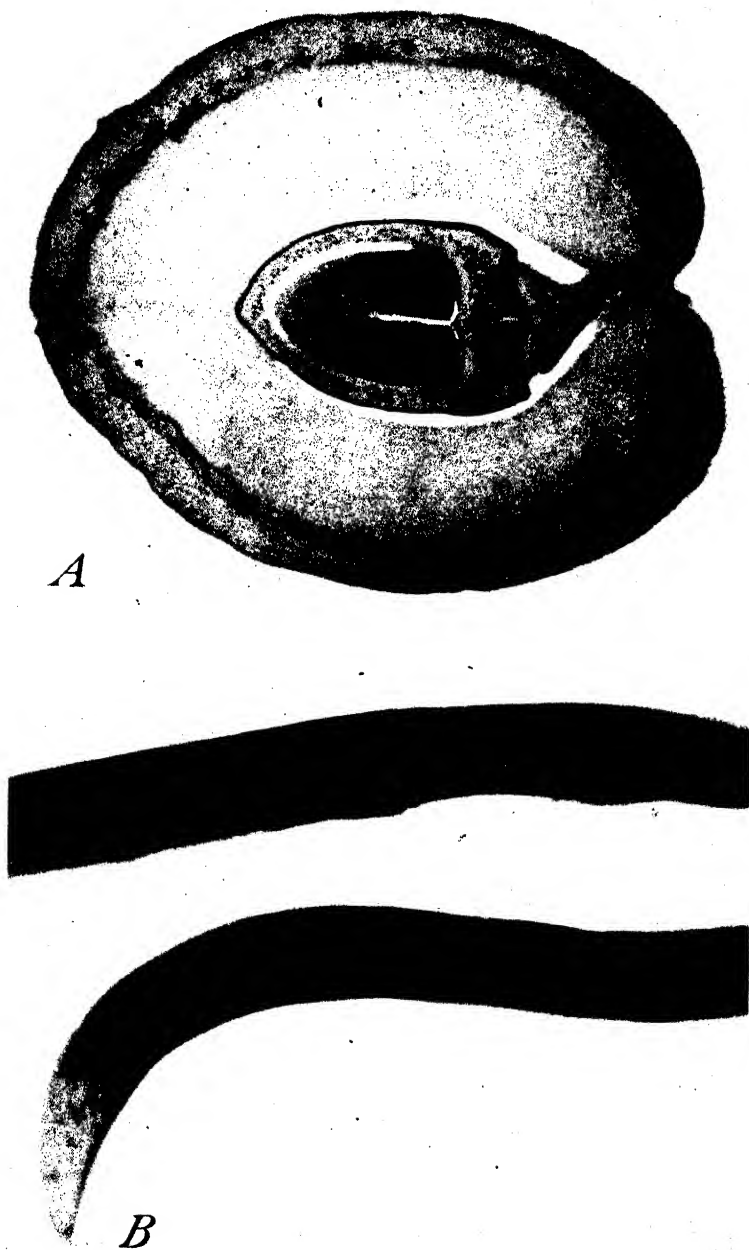
*A-B*, Transverse sections of roots showing distribution of black root symptoms; vascular discoloration is confined largely to the phloem and cambium, though in certain areas the rays and xylem parenchyma are discolored. *A* about  $\times 6$  and *B* about  $\times 70$ . *C*, An area of *B* under higher magnification, showing details of cellular involvement. Note particularly certain initial stages of suberization in the walls of young sieve tubes and fibers directly above the complete xylem vessel. The walls of these elements are normally composed of cellulose. About  $\times 750$ . *D*, Longitudinal section of a root showing symptoms of black root in the phloem and cambium. Slime bodies and the disorganized protoplasm show well, as do the suberized walls of diseased elements. Because they stain pale blue, the callose pads do not show well. About  $\times 750$ .



*A-B*, Transverse sections of the lower hypocotyl showing distribution of black root symptoms. *A* about  $\times 6$  and *B* about  $\times 70$ . *C*, An area in *B*, under higher magnification showing details of cellular involvement. Note particularly the collapse of cells near the cambium, the walls of which are not heavily suberized. In other areas the intensity of discoloration makes it appear that cellular structure has been obliterated. About  $\times 400$ . *D*, Longitudinal section of an area of the lower hypocotyl showing details of cellular involvement in the cambium and phloem. Here again callose pads are indistinct due to their staining reactions. About  $\times 400$ .



*A-B*, Transverse sections of the above-ground stem showing the distribution of black root symptoms. The phloem, except for mature fibers, and the cambium are completely necrosed. *A* about  $\times 25$  and *B* about  $\times 70$ . *C*, Tangential section through the stem showing early symptoms of black root. About  $\times 750$ .



*A*, Transverse section of a pod in the snap bean stage showing distribution of black root symptoms. About  $\times 16$  (or  $\times 25$ ). *B*, Unstained, diseased bean pods, cleared to show the extent of vascular discoloration. About  $\times 5$ .

that the deposits grow in thickness as the protoplasm degenerates until finally, in later stages, the walls are quite thick. Certain cells with less heavily suberized walls near the cambium are completely collapsed due perhaps to growth stresses of the surrounding healthy tissues, but no evidence of dissolution of cell walls has been found. The necrosed areas are notably lacking in starches, proteins, and fats, as compared with the surrounding, apparently unaffected areas.

In longitudinal sections, particularly of the phloem, several additional features can be seen to advantage. Normal cell constituents such as nuclei and slime bodies (Schleimklumpen of Strasburger) are particularly evident in the normal-appearing cells. In the diseased cells various degrees of involvement, as exemplified by protoplasmic disorganization, are evident (pl. 1, *D*). In certain sieve tubes, some of which show no evidence as yet of protoplasmic disorganization, callose pads are frequently found blocking the pores of the sieve plates. Sieve tubes thus affected recall a similar situation in perennial plants in which callose pads normally are present in the sieve tubes during the dormant period (3). Since the bean plant is an annual and does not, therefore, normally undergo a dormant phase, the writer is inclined to attribute the presence of callose pads in its sieve tubes to the activity of the virus. That no dormant phase normally exists in beans during the period in which the material was collected was obvious from examination of normal tissue. Physiologically, the presence of callose pads must greatly influence the transport of vital substances through the phloem. It could not be determined whether their formation precipitates the later-appearing degenerative phenomena or whether they are but coincidental with the beginning of a series of irreversible reactions that ultimately result in complete protoplasmic disorganization in affected cells. Chain reactions would seem to play a part in the sequence of events.

#### CAMBium

In all instances in which the phloem shows disease symptoms, the contiguous cambium likewise is affected. This generalization holds true not only for the root but for all portions of the plant in which a functional cambium has been found. Staining reactions identical with those described for phloem are evident in diseased portions of cambium. This is strictly true, however, only insofar as the meristematic zone immediately adjacent to the phloem can be regarded as cambium. The writer fully realizes the difficulty of defining a single row of meristematic cells as cambium to the exclusion of meristematic gradients contiguous on one side with phloem and on the other with xylem. However, those cells, not yet differentiated, lying closest to the diseased phloem show all gradations of suberization of cell walls and protoplasmic degeneration. Those nearest the phloem show symptoms identical with those of phloem, while those nearest the xylem exhibit symptoms typical of diseased xylem elements. In most instances, the cambial derivatives appear to undergo fewer divisions and to begin to differentiate much earlier in diseased areas than in areas relatively or entirely free from symptoms. Many cells with heavily suberized walls remain distinct, while others, usually showing deposits of less thickness, undergo almost complete collapse.



## XYLEM

Transverse and longitudinal sections of xylem tissue show very few symptoms of disease except, as stated above, in the ray parenchyma and in the xylem parenchyma immediately adjacent to certain vessels nearest the cambium. In a few instances, gum has been demonstrated in the lumen of certain young vessels, but this is exceptional. Likewise, very few tyloses were seen. Suberin deposits are present on the walls of badly diseased xylem parenchyma. The contents of diseased xylem and ray parenchyma show all gradations of disorganization and gumlike inclusions, just as in the phloem (pl. 1, B, C). The vessels are scalariform (as can be seen in longitudinal section) (3). In many instances the pores in a given view were definitely plugged by small deposits of gum, but no evidence is available to indicate how extensively the pores in a given vessel, or in a series are plugged. However, as will be brought out later, it is hardly necessary to find or postulate complete vessel plugging to account for the failure of the xylem as a conductive system.

## HISTOLOGY OF THE HYPOCOTYL

For the purposes of this study the hypocotyl is regarded critically, for it is in this area that practically all the transition of the vascular arrangement from root type to stem type takes place. Since there is, relatively, a greater amount of differentiated vascular elements in the hypocotyl than in either root or stem, it is not surprising that one finds the diseased areas more conspicuous here and more uniformly distributed than in either the root or stem.

Here, however, the necrotic areas are confined for the most part to the phloem and cambium, as was true in the root. Instances of ray penetration and involvement of xylem parenchyma are evident, but proportionately about as in the root (pl. 2). In several instances it appears that the cambium may be killed more quickly than the root.

Individual diseased cells, whether in phloem, cambium, or xylem, give identical staining reactions and distribution as do like cells from the root.

## HISTOLOGY OF THE STEM

As noted in a former publication (6), black root symptoms on the stem are not uniform, but reveal their presence by a dark streak or streaks running lengthwise of the stem. Other symptoms such as wilting, chlorosis, and mosaic patterns, in the leaves are also present, but since these are common to several virus diseases of beans, stem streaking is regarded as a better diagnostic symptom.

Transverse and longitudinal sections show relatively smaller numbers of necrotic areas in the stem than are present in either root or hypocotyl; but closer inspection indicates that relatively more necrotic cells occur in given areas of the stem than in comparable areas elsewhere (pl. 3). It also appears that protoplasmic disorganization is more sudden and complete in the stem than elsewhere, as fewer transitional stages are to be seen in tissue of comparable age, and, supposedly, comparable as to the time of initial infection.

The distribution of necrotic areas and the completeness of protoplasmic disorganization in areas of the stem are definitely correlated with the percentage of leaves and fruiting pedicels that individually show no symptoms of disease for various periods of time after other

parts are wilted and dead. Whether initial infection of a given plant occurred through the above-ground parts or through the roots could not be ascertained from the present study. The histological evidence indicates, however, that the transitional area of the hypocotyl has an important relationship to the amount and distribution of necrosis in both the stem and root.

However, the individual necrotic areas of the stem, like those of the root and hypocotyl, are confined almost exclusively to the phloem and cambium. Nevertheless, as in other organs of the plant, protoplasmic disorganization often extends for short distances down the rays and into the xylem parenchyma surrounding the vessels contiguous to the cambium. Here, as elsewhere, certain vessels contain various amounts of gum, and the question arises again as to how completely the scalariform perforations are plugged.

#### HISTOLOGY OF THE POD

Although the only exterior symptoms of black root on the pods consist of a slight darkening of one or both sutures, accompanied by a slight pitting of the epidermis in older pods (6), transverse sections show that necrosis of the vascular system is quite extensive (pl. 4, A). The largest vascular bundles of the pod lie below the sutures. No doubt the size of these suture bundles, with their greater number of phloem elements, rather than the distribution of necrotic areas, is responsible for the expression of symptoms exteriorly only along the sutures. It is evident that essentially all the vascular tissue shows necrosis, but since the smaller bundles contain so little phloem, the intensity of discoloration is not sufficient to show through the chlorenchyma of fresh pod walls. It is thought, however, that the involvement of the smaller bundles is responsible for the pitting of the pods, as the pits appear to be the result of collapse of desiccated tissues, particularly in the vicinity of stomata. When the chlorophyll is extracted and the pods appropriately cleared, the extent of vascular necrosis is evident even in unstained preparations (pl. 4, B).

Phloem necrosis also extends from the ventral vascular (suture) bundles down through the funiculi into the seed. Evidence of suberization of cell walls and protoplasmic disorganization is evident to some extent in certain parenchyma (transfusion) areas along the smaller veins. In no instance was there evidence of vascular necrosis in the embryo, although considerable areas were found in the seed coats at the chalaza. This observation raises an interesting point in regard to the mechanics of seed transmission of black root. It is, of course, conceivable that the virus might pass through the transfusion areas of the seed coat at the chalaza and thereby reach the vascular system of the embryo. It is also conceivable that embryo infection takes place during germinative stages of the seed, since the enlarging embryo in rupturing the seed coat might release sufficient inoculum from the phloem of the seed coat to bring about infection. This viewpoint presupposes that the virus remains active in the seed coat, a point that has not as yet been investigated. However, we do know that under certain conditions a small percentage of the disease is seed borne (6). Both these hypotheses are contrary to findings of other investigators (2), but they were working with a disease which is not seed transmitted.

## DISCUSSION

Plants affected with various virus diseases have been studied anatomically but of those characterized by necrosis, the reports on location, pattern, and appearance of the areas are rather uniformly in agreement as to certain critical details (1, 2, and perhaps 4).

Results of the present study have afforded an histological explanation for the odd distribution of symptoms produced by black root on snap beans. Apparently being confined largely to the phloem and cambium of the root, hypocotyl, stem, and pods of a given plant, the number of necrotic areas present seems definitely correlated with the amount and extent of symptoms that the plant exhibits. This observation seems particularly well borne out in the hypocotyl, where invariably the amount of necrotic tissue is relatively greater than in either root or stem of the same plant.

The limited extent to which the xylem tissue is involved and the fact that extensive gum or tylosis formation does not occur in the vessels indicates that factors other than necrosis are responsible for the rapid wilting of diseased plants in the presence of abundant moisture. Aside from the plugging of the sieve tubes by callose pads, which doubtless influences the nutrition of the plant, the writer feels that sudden wilting can best be explained histologically through the reaction of the virus on the xylem parenchyma. Even though only the parenchyma surrounding the outermost vessels, i. e., those contiguous to the cambium, is apparently affected, the result is the effectual and sudden wilting of the plant. This explanation can best be accepted by postulating the existence of a very rapid functional gradient between the old vessels and those that have recently differentiated (3), as well as assigning to the living xylem parenchyma an important influence on water conduction. Such an interpretation apparently was considered by Weimer (9) in his work on alfalfa dwarf. He found that when relatively inconsiderable numbers of xylem vessels contiguous to the cambium were plugged in the alfalfa roots the plants wilted. By pumping water through diseased and healthy roots he demonstrated that very little water passed through those roots in which the xylem vessels contiguous to the cambium were plugged. It is also conceivable that injury to the xylem parenchyma was a considerable factor in the wilting of the living alfalfa plants.

The writer is also not unmindful of the possibility that the vessels might be plugged by small deposits of gum over the scalariform perforations between vessels, as mentioned earlier in this paper. This could be a particularly significant factor when one considers the great amount of anastomosis of the ducts, particularly in the transition areas of the hypocotyl. It is very difficult histologically to demonstrate with certainty the proportion of the scalariform perforations that are plugged by gum.

Finally, the physiological effects of the amount and severity of phloem and cambium necrosis alone, without regard to the xylem, could well upset the nutritional balance of the plant to the extent that sudden wilting would result. Elaborated foods are essential to a wide variety of energy relations concerned in the necessarily continuous growth and extension of the root system into its "absorption environment," as well as to the actual process of material absorption from the soil. Theoretically, the root system could operate fairly efficiently

under the stress of diminishing food supplies for various periods of time; but when it is no longer possible for its compensation mechanism to maintain functional equilibrium, the root system fails and the plant suddenly wilts. Histological evidence for this explanation is necessarily fragmentary, consisting chiefly of tests for proteins, fats, starch, etc., all of which were much depleted or absent in necrosed tissues. This, too, is in accord with the findings of Hill and Orton (4).

In regard to the histology of seed transmission of black root, it can be said only that symptoms of infection (necrosis of phloem elements) have been traced by the writer into the seed coat near the chalaza. Other workers (2) found the same situation in their work with curly top of beets. It so happens that curly top is not transmitted through the beet seed, while black root is seed-transmitted, at least to some extent. Others have shown that relatively few virus diseases are seed-transmitted, the outstanding example of positive transmission being common bean mosaic. The histological evidence being the same for a disease that is sometimes transmitted through the seed (black root) as for one which is apparently never transmitted through the seed (curly top), it can be concluded only that some factor unknown to the writer determines the mode and frequency of embryonic inoculation. Hypothetically, it is reasonable to assume that inoculation might occur when the vascular system of the seed coat is ruptured during germinative stages of the seed.

#### SUMMARY

Snap beans affected with black root have been examined histologically. This report is based on studies of both fresh and fixed material, appropriately stained, of several varieties and progenies from segregating hybrids.

The peculiar external manifestation of such symptoms as differential streaking and wilting of certain plant parts has been shown to be correlated histologically with differential necrosis of the phloem, cambium, and outermost layer of xylem in the root, hypocotyl, stem, and pod.

Necrotic symptoms in the phloem varied from slight cytoplasmic disorganization in those cells recently infected through stages of more or less complete destruction of the cytoplasm and nucleus. In the end phases the cell contents resembled gum, and the cellulose cell walls became covered by deposits of suberin. Callose pads were also noted in sieve tubes showing early symptoms of disease. Whether the effect of these initiate degenerative changes or are but one of a series of chain reactions, is not known. In that area of the cambial zone contiguous to the phloem conditions similar to those in phloem prevailed; while in the areas nearest differentiating xylem, the symptoms most nearly resembled those characteristic of xylem. In the vessels very little evidence of plugging was observed except in the scalariform perforations, and very few tyloses were seen. The parenchyma about the youngest vessels contiguous to the cambium was severely necrosed, and necrosis was occasionally found in the ray parenchyma.

It is tentatively suggested that the sudden wilting characteristic of black root may be due in great part to cessation of activity of the young vessels through destruction of the vital xylem parenchyma. The possibility of mechanical blocking of the scalariform perforations

by gum, particularly in the hypocotyl, as well as the probable effect of phloem and cambium necrosis on the energy relations of the root system, are also considered.

Necrosis of the phloem was observed to extend throughout the vascular system of the pod and through the bundles of the funiculi into the seed coat at the chalazal end of the seed. No evidence of infection was observed in the embryo, but only very young embryos were examined. It is suggested that seed transmission may be accomplished by transfusion of the virus from the seed coat to the embryo, or that inoculation may occur as the embryo ruptures the vascular system of the seed coat during germination.

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## FACTORS AFFECTING THE GERMINATION OF VARIOUS DROPSEED GRASSES (*SPOROBOLUS* SPP.)<sup>1</sup>

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### INTRODUCTION

At the request of the Soil Conservation Service, United States Department of Agriculture, a study of the optimum conditions for the germination of the seed of *Sporobolus* spp. was begun in 1935. Hoover (22)<sup>3</sup> states that these grasses can be used widely in erosion-control work because of their adaptation to the climatic and soil conditions of the Great Plains and the Southwest. In addition to the practical importance of the germination behavior of the seed of these plants, the material furnished an opportunity to study the diverse behavior of several species of a genus with an unusual and variable fruit structure. With some species there were enough samples to study the variability and differences in response to various treatments within the species.

### EARLIER INVESTIGATIONS

Guérin (16) reported that the strongly developed pericarp of *Sporobolus tenacissimus*, of *S. macrospermus*, and of *S. ciliatus* presents a very specialized structure in which almost the entire pericarp is transformed into mucilage of such a nature that under the influence of the least trace of water the seed is liberated. In *S. heterolepsis* the outer layers of the fruit coat are strongly sclerified, and only the inner zone is composed of mucilage. The seed coat in *Sporobolus* is considerably developed.

Jackson (23) stated that the seed coat of *Sporobolus* prevents water absorption, but no data were presented in support of the statement. She germinated the seed at constant temperatures of 25° or 35° C. on blotting paper in Petri dishes or in sandy loam. She found that shaking the seed with sand from 4 to 9 hours was of no benefit.

Blake (4) reported that seed of *Sporobolus asper* varied in response from year to year. She stated that the germination of the seed improved with age under the conditions she used.

Wilson (38), working with *Sporobolus strictus*, *S. cryptandrus*, *S. flexuosus*, *S. wrightii*, and *S. airoides*, found that with the exception of *S. airoides* the seeds did not germinate at all well without scarifying. He stated that the seed coats were almost impervious to water. He obtained over a 6-year period an average germination of 3.75 percent

<sup>1</sup> Received for publication January 28, 1941. Investigations conducted in the former Division of Seed Investigations, Bureau of Plant Industry, U. S. Department of Agriculture.

<sup>2</sup> Grateful acknowledgment is made to the following members of the Bureau of Plant Industry: To E. H. Toole, for suggestions and criticisms throughout these investigations; to Robert C. Hess, Jr., for assistance with the preparation of the material; to Albina F. Musil, for assistance in checking the seed identifications. Acknowledgment is also made to A. E. Brandt of the Soil Conservation Service for assistance with the statistical analysis.

<sup>3</sup> Italic numbers in parentheses refer to Literature Cited, p. 714.



for *S. cryptandrus*, 1.11 percent for *S. flexuosus*, 1.54 percent for *S. strictus*, 13.17 percent for *S. wrightii*, and 35.69 percent for *S. airoides*. The germination tests were made at a temperature alternation of 20° to 30° C.

The extensive literature on dormancy and on the physiology of germination will not be reviewed here, but pertinent papers will be mentioned in the discussion of results.

#### MATERIAL AND METHODS

The following species were included in this study: *Sporobolus cryptandrus* (Torr.) A. Gray, sand dropseed; *S. flexuosus* (Thurb.) Rydb., mesa dropseed; *S. contractus* Hitchc., spike dropseed; *S. giganteus* Nash, giant dropseed; *S. airoides* (Torr.) Torr., alkali sacaton; *S. wrightii* Munro., sacaton; and *S. asper* (Michx.) Kunth. The seed was furnished by M. M. Hoover of the Soil Conservation Service from various regional plantings and from collections of native plants, and by the Division of Forage Crops and Diseases, Bureau of Plant Industry. Also one sample (No. 769232) was collected from wild plants by Mary Thayer, of the Seed Laboratory, Kansas State College of Agriculture and Applied Science. The origin, date of collection, and other historical data on the samples are given in table 1.

After receipt, the seed was stored in the laboratory at room temperature. The seed as received was cleaned by means of an air-blast blower and sieves. Only fully developed caryopses were used in the study, but in these were sometimes included seed of varying sizes. The "seed" as referred to in this paper includes the true seed with attached pericarp and, in some cases, with the lemma and palea. The seed was put to germinate in Petri dishes on paper toweling moistened with tap water or with a 0.2-percent solution of potassium nitrate, at various constant and alternating temperatures. The alternation of temperatures was secured by transferring the test from one chamber to another. The test was at the first temperature listed for 17 hours, and at the last temperature for 7 hours daily. In order to obtain light in combination with a high temperature an alternation of room temperature to 35° C. was used. The tests were placed in the north window in an air-conditioned room from 4 p. m. until 9 a. m. and at 35° from 9 a. m. until 4 p. m. The room temperature varied between 16° and 24° but was maintained at near 20° much of the time except during the summer. The 20° to 30° alternation, with light exposure, was obtained by using a specially built daylight germinator for the 30° temperature.

To prechill, the seed was placed on the moistened paper toweling in the Petri dishes and held at 3° C. for the designated number of days prior to being placed at the germination temperature. The temperature of the 3° chamber varied between 2° and 5°. The date of counts was calculated from the day the seed was placed to prechill.

For sulfuric acid treatments, the seed was placed in a small porcelain crucible, covered with an excess of the acid, and stirred constantly for various lengths of time, after which it was washed in running tap water for 30 to 45 minutes and dried thoroughly before being tested for germination. The concentrated sulfuric acid used had a specific gravity of 1.84 and was of at least 94-percent concentration. Sulfuric acid approximately 71 percent by volume (approximately 80

TABLE 1.—Historical data on all samples of seed of *Sporobolus* spp.

Sample No.	Senders' identification No.	Approximate purity	Species	Place of collection	Year or date of collection	Date received	Notes by collector
757544	WY341-251	Percent	<i>Sporobolus cryptandrus</i>	Cheyenne, Wyo.	Oct. 1, 1938	Mar. 23, 1935	Sandy soil.
101	101	86	<i>Sporobolus cryptandrus</i>	Evans, Colo.	Sept. and Oct. 1937	Nov. 12, 1938	From vacant lots.
769004	100	97	<i>Sporobolus cryptandrus</i>	Amarillo, Tex.	Sept. 19, 1938	Dec. 17, 1937	From same lots as 769004.
102	100	98	<i>Sporobolus cryptandrus</i>	do.	Aug. 1938	Nov. 5, 1938	From high plains soils on abandoned farmlands.
1296	1256	98	<i>Sporobolus cryptandrus</i>	Perryton, Tex.	1935	Nov. 12, 1938	do.
772727	AS10	93	<i>Sporobolus cryptandrus</i>	O'Neill, Nebr.	1935	Mar. 31, 1938	Grown under irrigation.
769223	AS10	83	<i>Sporobolus cryptandrus</i>	Tucson, Ariz.	June 1937	Aug. 24, 1938	Nursery stock.
AS10	AS10	88	<i>Sporobolus cryptandrus</i>	do.	1938	Feb. 7, 1939	Grown under irrigation.
769203	OS18	83	<i>Sporobolus cryptandrus</i>	Seiling, Okla.	Aug. 1937	Dec. 17, 1937	Nursery stock.
01949	O-1949	98	<i>Sporobolus cryptandrus</i>	do.	Sept. 20, 1938	Nov. 12, 1938	Abandoned fields and along roadways.
772728	772728	98	<i>Sporobolus cryptandrus</i>	do.	1936	Mar. 31, 1938	do.
772728	772728	90	<i>Sporobolus cryptandrus</i>	Pullman, Wash.	1935	do.	do.
767594	52	93	<i>Sporobolus cryptandrus</i>	Morgan, Utah	Jan. 1938	Feb. 23, 1938	do.
769222	55	82	<i>Sporobolus cryptandrus</i>	Madhattan, Kans.	Aug. 18, 1938	Aug. 26, 1938	Vacant lot.
767742	55	75	<i>Sporobolus contractus</i>	Morgan, Utah	Jan. 1937	Feb. 23, 1938	Nursery stock.
767287	AS10	84	<i>Sporobolus contractus</i>	Tucson, Ariz.	June 1937	Jan. 10, 1938	Roadside near nursery. Seeds matured in late summer.
AS763	AS763	98	<i>Sporobolus contractus</i>	do.	Jan. 26, 1939	Feb. 7, 1939	Seeds matured in late summer. Stored on plants till collected. Nursery stock.
AS932	AS932	96	<i>Sporobolus giganteus</i>	do.	Oct. 21, 1938	do.	do.
767281	A923	74	<i>Sporobolus giganteus</i>	Wilcox, Ariz.	1934	Jan. 10, 1938	do.
767283	A9081	36	<i>Sporobolus flavus</i> 1	do.	Oct. 1936	Feb. 10, 1938	Nursery stock.
AS961	A9081	98	<i>Sporobolus flavus</i> 2	Tucson, Ariz.	Sept. and Oct. 1938	Feb. 7, 1939	Nursery stock.
767280	A10117	53	<i>Sporobolus flavus</i> 1	Wilcox, Ariz.	Nov. 20, 1937	Jan. 10, 1938	do.
767281	16	63	<i>Sporobolus flavus</i> 2	Leeds, Utah	Feb. 11, 1938	Feb. 23, 1938	do.
767741	53	90	<i>Sporobolus flavus</i> 2	Morgan, Utah	Jan. 1938	do.	do.
767716	31	53	<i>Sporobolus airoides</i>	Leeds, Utah	Feb. 11, 1938	do.	do.
767700	3-U, No. 2	86	<i>Sporobolus airoides</i>	Price, Utah	Jan. 24, 1938	do.	do.
772724	AS501	97	<i>Sporobolus airoides</i>	Southcentral Kansas	1936	Mar. 31, 1938	do.
AS501	AS501	98	<i>Sporobolus airoides</i>	Winslow, Ariz.	Nov. 1936	Feb. 7, 1939	Grown under irrigation.
769224	A666	79	<i>Sporobolus erioglossi</i>	Tucson, Ariz.	Nov. 1937	Aug. 24, 1938	Nursery stock.
A666	A666	98	<i>Sporobolus erioglossi</i>	do.	Aug. 1938	Feb. 7, 1939	do.
772725	772725	89	<i>Sporobolus asper</i>	do.	1937	Mar. 31, 1938	do.

<sup>1</sup> Probably a mixture; some seeds smooth, like *S. cryptandrus*, most of them striate, like *S. contractus*.

<sup>2</sup> Not very sure of identification of this group. Apparently contains some *S. cryptandrus*.

percent by weight) was secured by diluting 3 volumes of acid with 1 volume of distilled water.

The sprouted seeds were counted on the third, fifth, and seventh days after being placed at germination temperatures and at 7-day intervals thereafter. It was necessary to count the sprouts often during the first week, especially in tests with treated seed, because germination was rapid under proper conditions. Seeds that produced a normally developed root and shoot were considered as germinated.

The germination values summarized in the various tables are means based on duplicate tests of 100 seeds each. In tables 2, 5, 6, 14, 15, and 16, half percents have been raised to the next higher percentages. In tables 3, 4, 8, and 11, value for error and tests of significance have been calculated by the analysis-of-variance method as adapted by Snedecor (31). Differences have been designated significant when the probability is equal to or less than 5 percent and highly significant when equal to or less than 1 percent. Mean squares marked with an asterisk (\*) are significant with reference to error and marked with a double asterisk (\*\*) are highly significant with reference to error.

## EXPERIMENTAL RESULTS

### SPOROBOLUS CRYPTANDRUS

#### GERMINATION OF INTACT SEEDS

Five samples of seed of *Sporobolus cryptandrus* were tested at a wide range of constant and alternating temperatures, in some cases with definite light exposure, in order to determine the general response to temperature and light. The effect of potassium nitrate in the germination medium was also determined. Results are given in table 2. In general, germination was best at the room temperature to 35° C. alternation, in which the seed received some light from a north window while at a room temperature varying around 20°. When the seed had no pretreatment, room temperature to 35° alternation, with one exception, was definitely superior to any combination that included a temperature continuously above 20° for the longer period or above 35° for the shorter period of the alternation. Germination at 15° to 35° alternation was somewhat better than at 20° to 35°, and at either of these temperatures it was much superior to 20° to 30° alternation with light. Constant temperatures produced negligible results, but 35° constant was superior to 10°, 15°, 20°, 25°, or 30°. Potassium nitrate was beneficial, especially at the more favorable temperatures.

In order to determine more definitely the effect of light at room temperature to 35° C. alternation during May 1936, a check test of sample No. 757544 was placed in a tin box to exclude all light. (Results are not given in tabular form.) Germination with light excluded was 15 percent with nitrate and 10 percent with water, compared with 67 percent with nitrate and 31 percent with water on the test exposed to light. The results at room temperature to 35° with light excluded were comparable to those at 20° to 35° (24 percent with nitrate and 8 percent with water).

With some samples a much higher germination percentage was obtained after the seed had been prechilled at 3° C. A more detailed study was made of the effect of prechilling, the results of which will be presented later. Results not given in the tables indicated that storing

TABLE 2.—*Germination in 49 to 56 days of five samples of seed of Sporobolus cryptandrus at various temperatures*

Prechilling period (days)	Germination temperature (° C.)	Germination in tests begun on indicated date with specified treatment of sample No.—													
		757544						766903		766904		769223		769232	
		May 10, 1935		Nov. 22, 1935		March 26, 1936		Jan. 13, 1938		Jan. 19, 1938		Sept. 7, 1938		Sept. 12, 1938	
		Potassium nitrate	Water	Potassium nitrate	Water	Potassium nitrate	Water	Potassium nitrate	Water	Potassium nitrate	Water	Potassium nitrate	Water	Potassium nitrate	Water
		Pct.	Pct.	Pct.	Pct.	Pct.	Pct.	Pct.	Pct.	Pct.	Pct.	Pct.	Pct.	Pct.	Pct.
0.	10.	0	0												
0.	15.	1	1												
0.	20.	1	0												
0.	25.	0	0												
0.	30.	3	1												
0.	35.	21	3												
0.	10 to 25.	11	2												
0.	15 to 25.	4	2												
0.	10 to room.	2	1												
0.	10 to 30.	25	4												
0.	10 to 35.			55	25										
0.	15 to 35.			77	49	62	34	77	42	19	12				
0.	25 to 15.	2	0												
0.	35 to 10.	11	6												
0.	20 to 30 with light.	2	1	27	13	10	9	33	16	3	1	5	2	20	6
0.	20 to 35.	9	13	43	18	10	4	67	26	10	5	16	10	78	27
0.	Room to 35.	54	29	70	43	93	76	93	56	61	12	13	7	87	25
0.	20 to 40.							66	40	16	5	19	6	70	23
0.	25 to 40.							57	28	7	3				
7.	20 to 35.			42	28	16	7	90	72	24	17				
7.	Room to 35.			50	18	88	76	97	83	51	16				
14.	15 to 35.							85	77	25	10				
14.	20 to 35.			68	41	43	12	97	83	36	13	70	51	86	50
14.	Room to 35.	69	27	80	46	97	70	92	75	50	19	75	51	90	57
14.	15 to 35.							93	86	52	31				
21.	20 to 35.			81	70	45	40	93	81	44	13				
21.	Room to 35.	72	35	68	25	91	85	92	79	55	17				
21.	15 to 35.							80	84	48	22				
28.	20 to 35.			99	85	80	77	87	82	54	27	85	88	86	58
28.	Room to 35.	85	63	98	73	96	86	97	86	38	25	87	85	88	67
28.	20 to 30.			44	17	51	36								
28.	30.			63	33	34	34								
28.	35.			68	63	69	73								
28.	15 to 35.							87	84	53	28				
56.	20 to 35.			88	86	96	90								
56.	Room to 35.			93	70	90	87								

the seed dry at 2° did not have the effect obtained by prechilling moist seed at 3°.

Although with some samples practically complete germination of the viable seeds was obtained at room temperature to 35° C. alternation when the substratum was moistened with potassium nitrate, other samples appeared to be resistant to the combined effects of temperature, light, and nitrate and to require additional treatment. In later work on pretreatment of the seed, room temperature to 35° alternation was used as the standard temperature, but the 20° to 35° alternation was continued as a check, since it approximated the same temperature range with a better control of the lower temperature.

#### TREATMENT WITH SULFURIC ACID

In order to determine the possible effect of the seed covering in restricting germination, seed was treated with sulfuric acid. Preliminary trials with concentrated sulfuric acid (94 percent) caused

injury to the seed, and germination results were low. Untreated seed and seed treated for 1, 2, 5, 7, and 9 minutes with approximately 71-percent sulfuric acid were germinated with potassium nitrate and with water at room temperature to 35° C. alternation and at 20° to 35° alternation. The germination results with 8 samples, untreated and treated for 2 and 5 minutes, are given in table 3.

In this, as well as in subsequent tables, the variance for duplicates was very low in comparison with the variance for sample interaction, indicating uniformity of conditions of germination.

There was a very highly significant difference between the mean results for the two germination-temperature treatments and between potassium nitrate and water. Pretreatment with approximately 71-percent sulfuric acid overcame the resistance to germination. A 5-minute acid treatment was significantly better than a 2-minute acid treatment. In general, however, treatment for 2 minutes was sufficient when followed by germination at room temperature to 35° C. alternation with the use of potassium nitrate. This length of treatment was not injurious even to those samples that germinated well without treatment. Sample No. 272727 required a longer treatment (5 or even 7 minutes) for maximum germination. Five-minute treatment caused injury to sample No. O-1949, which germinated well without treatment at the optimum conditions. In all cases tried, treatment for 9 minutes was injurious. The highly significant interaction between the germination temperatures and the acid treatments is brought about by the very low results for the untreated seed at the less favorable temperature. In a few trials at the optimum condition a 1-minute acid treatment was almost as good, or as good, as the 2-minute treatment.

TABLE 3.—Germination in 28 days of seed of *Sporobolus cryptandrus* at 2 temperature alternations after treatment with approximately 71-percent sulfuric acid, November 1938

ORIGINAL DATA									
Treatment of substratum and sample No.	Observations	Germination after acid treatment for indicated period at—							
		Room temperature to 35° C.				20° to 35° C.			
		0 min-ute	2 min-utes	5 min-utes	Mean	0 min-ute	2 min-utes	5 min-utes	Mean
Potassium nitrate:	Number	Percent	Percent	Percent	Percent	Percent	Percent	Percent	Percent
766904.....	2	67.5	97.5	94.0	86.33	10.5	95.0	89.0	64.83
100.....	2	53.5	95.0	99.5	82.67	5.5	75.5	94.5	58.50
1256.....	2	55.5	97.5	89.0	80.67	20.0	90.0	87.5	65.83
272727.....	2	54.5	63.5	85.5	67.83	5.0	57.5	61.0	41.17
769223.....	2	30.0	78.5	78.5	62.33	15.5	52.0	81.5	49.67
101.....	2	90.0	96.5	99.0	95.17	6.5	76.0	95.0	59.17
769232.....	2	91.0	91.5	89.0	90.50	87.0	91.5	86.5	88.33
O-1949.....	2	98.0	99.5	78.0	91.83	62.0	93.5	68.0	74.50
Total or mean.....	16	67.50	89.93	89.06	82.16	26.50	78.87	82.87	62.75
Water:	Number	Percent	Percent	Percent	Percent	Percent	Percent	Percent	Percent
766904.....	2	27.5	87.5	90.5	68.50	9.0	79.5	87.0	58.50
100.....	2	9.5	44.0	85.5	46.33	2.5	32.5	47.0	27.33
1256.....	2	21.5	86.0	86.0	64.50	5.0	65.0	68.5	46.16
272727.....	2	27.5	22.0	48.5	32.67	0.5	14.5	37.5	17.50
769223.....	2	16.0	50.5	78.5	48.33	14.5	31.5	69.5	38.50
101.....	2	68.5	71.5	84.5	74.83	3.0	39.5	66.5	36.33
769232.....	2	48.5	75.0	71.5	65.00	15.0	76.0	72.0	54.33
O-1949.....	2	65.5	97.0	73.0	78.50	17.5	86.0	86.0	56.50
Total or mean.....	16	35.56	66.69	77.25	59.83	8.37	53.06	64.25	41.89
Grand total or mean.....	32	51.53	78.31	83.15	70.99	17.43	65.96	73.56	52.32

TABLE 3.—*Germination in 28 days of seed of Sporobolus cryptandrus at 2 temperature alternations after treatment with approximately 71-percent sulfuric acid, November 1938—Continued*

ORIGINAL DATA—Continued

Treatment of substratum or germination temperatures	Total observations	Mean germination after acid treatment for—			
		0 minutes	2 minutes	5 minutes	Mean
	Number	Percent	Percent	Percent	Percent
Potassium nitrate.....	32	47.00	84.40	85.96	72.45
Water.....	32	21.96	59.87	70.75	50.86
Room temperature to 35° C.....	32	51.53	78.31	83.15	70.99
20° to 35° C.....	32	17.43	65.96	73.56	52.32
All treatments.....	64	34.48	72.13	78.35	61.65

## ANALYSIS OF VARIANCE FOR ALL TREATMENTS

Source	D. F.	Mean square	F
Between samples.....	7	3,869.04	-----
A (2 v. 5 minutes).....	1	1,237.53	2.65
B (2+5 minutes v. twice 0 minutes).....	1	70,905.01	**152.15
C (KNO <sub>3</sub> v. H <sub>2</sub> O).....	1	22,381.92	**48.03
D (temperature <sub>1</sub> v. temperature <sub>2</sub> ).....	1	16,744.00	**35.93
B×D.....	1	5,704.17	**12.24
Other interactions.....	6	335.48	-----
Error (sample X treatments).....	77	466.01	-----
Between duplicates.....	96	26.39	-----
Total.....	191	-----	-----

## ANALYSIS OF VARIANCE FOR 2- AND 5-MINUTE ACID TREATMENTS

Between samples.....	7	2,773.68	-----
A (2 v. 5 minutes).....	1	1,237.53	*4.10
C' (KNO <sub>3</sub> v. H <sub>2</sub> O).....	1	12,640.50	**41.82
D' (temperature <sub>1</sub> v. temperature <sub>2</sub> ).....	1	3,850.03	**12.77
Interactions.....	4	241.54	-----
Error (sample X treatments).....	49	301.55	-----
Between duplicates.....	64	26.53	-----
Total.....	127	-----	-----

## COMPARISON OF PRECHILLING AND ACID TREATMENTS

The germination results of eight samples after prechilling for 14, 28, and 56 days at 3° C. and after a 2-minute treatment with approximately 71-percent sulfuric acid are given in table 4. Prechilling for 28 or 56 days was superior to prechilling for 14 days. Taken as a whole, the 2-minute treatment with acid was significantly superior to the prechilling treatments. With either prechilling or acid treatment, the use of potassium nitrate gave increased germination.

## SIZE OF SEED AND GERMINATION

The cleaned seed of sample No. 769223 was quite variable in size. On September 7, 1938, a portion of the sample was passed through sieves, and the different seed sizes were germinated separately. The results are given in table 5. The results after prechilling represent the full value of the sample for the different groups as the seed either germinated or decayed. The smaller seed had less potential viability than the larger and was benefited less by prechilling, indicating a less resistant seed coat. This was the only sample of *Sporobolus cryptandrus* studied that did not have a potential germination value of more

than 90 percent. When the smaller seed had been sieved out, the germination value of the remainder of the sample was above 90 percent.

TABLE 4.—Germination in 84 days with room temperature to 35° C. alternation of eight lots of *Sporobolus cryptandrus* seed prechilled at 3° for various periods or treated with approximately 71-percent sulfuric acid for 2 minutes, November 1938

## ORIGINAL DATA

Sample No.	Observations	Germination of seed with potassium nitrate after indicated treatment					Germination of seed with water after indicated treatment				
		Prechilled for—			Acid-treated for 2 minutes	Mean	Prechilled for—			Acid-treated for 2 minutes	Mean
		14 days	28 days	56 days			14 days	28 days	56 days		
	Number	Percent	Percent	Percent	Percent	Percent	Percent	Percent	Percent	Percent	Percent
766904	2	86.5	82.5	82.0	97.5	87.12	65.5	78.5	80.0	87.5	87.87
100	2	36.0	56.5	63.5	95.0	62.75	10.0	13.0	21.0	44.5	22.12
1256	2	62.5	81.0	86.5	97.5	81.87	31.0	42.5	61.5	86.0	55.25
272727	2	74.0	71.5	96.0	67.5	77.25	57.0	62.0	79.0	23.5	55.37
769223	2	83.0	85.5	74.5	80.5	80.87	45.0	89.0	87.5	62.5	71.00
101	2	81.5	87.0	74.0	96.5	84.75	44.0	56.0	20.0	71.5	50.12
769232	2	91.0	90.5	93.0	92.0	91.62	85.0	84.0	84.0	75.5	82.37
O-1949	2	92.0	100.0	90.5	99.5	95.50	50.0	81.5	71.0	97.0	74.87
Total or mean	16	75.81	81.81	82.50	90.75	82.72	48.44	63.44	64.12	68.50	61.12

Treatment of substratum	Total observations	Mean germination for all samples at indicated treatment				
		Prechilled for —			Acid-treated for 2 minutes	
		14 days	28 days	56 days		
Potassium nitrate and water	Number 32	Percent 62.12	Percent 72.62	Percent 73.31	Percent 79.62	Percent 71.91

## ANALYSIS OF VARIANCE FOR ALL TREATMENTS

Source	D. F.	Mean square	F
Between samples	7	3,345.23	
A (KNO <sub>3</sub> v. H <sub>2</sub> O)	1	14,921.28	**34.34
B (Prechilling v. acid)	1	2,531.76	*5.83
C (14 v. 56 days prechilling)	1	2,002.56	*4.61
D (14+56 v. (2) (28) days prechilling)	1	513.52	1.18
Treatment interaction	3	145.53	
Error (sample×treatment)	49	434.45	
Between duplicates	64	28.95	
Total	127		

## ANALYSIS OF VARIANCE FOR PRECHILLED TREATMENTS

Source	D. F.	Mean square	F
Between samples	7	3,573.32	
A (KNO <sub>3</sub> v. H <sub>2</sub> O)	1	10,965.37	**39.94
C' (14 v. 56 days prechilling)	1	2,002.56	*7.29
D' (14+56 v. (2) (28) days prechilling)	1	513.52	1.87
Treatment interactions	2	216.00	
Error (sample×treatment)	35	274.58	
Between duplicates	48	26.19	
Total	95		

TABLE 5.—*Germination in 49 days of seed of Sporobolus cryptandrus, sample 769223, grouped according to seed size, September 1938*

Sieve size (mm.) <sup>1</sup>	Germination of seed at room to 35° C. temperature alternation			
	Not prechilled		Prechilled 28 days	
	Germinated with potassium nitrate	Germinated with water	Germinated with potassium nitrate	Germinated with water
	Percent	Percent	Percent	Percent
1.016.....	25	3	97	98
0.686.....	35	12	91	98
0.610.....	32	3	84	89
0.508.....	24	18	54	45
Mean.....	29.0	9.0	81.5	82.5

<sup>1</sup> The seed passed through the indicated sieve size and was retained by the next smaller.

## REGIONAL OR SAMPLE DIFFERENCE

There were differences in the degree of resistance to germination among the different samples of *Sporobolus cryptandrus* studied. Samples from the same State were of similar behavior. Samples from Texas, Arizona, Nebraska, and Wyoming did not give maximum germination at room temperature to 35° C. alternation with light and nitrate, whereas samples from Colorado, Oklahoma, Washington, Utah, and Kansas gave maximum germination under these conditions (table 6). All *S. cryptandrus* samples studied (except No. 769223) had a potential germination value of 90 percent or above. The States furnishing samples of seed having similar behavior do not form regional groups; however, it is believed that the resistant samples came from localities with semiarid conditions. Samples that germinated promptly at room temperature to 35° alternation with nitrate are referred to in this paper as "nonresistant," and samples that required additional treatment as "resistant."

TABLE 6.—*Germination in 49 days of Sporobolus cryptandrus seed of various ages collected in different States*

Sample No.	State where sample was collected	Date of germination test	Approximate age of seed	Germination, with indicated treatment, at—					
				20° to 35° C.		Room to 35° C.		Room temperature to 35° C. (after prechilling at 3° for 28 days)	
				Potassium nitrate	Water	Potassium nitrate	Water	Potassium nitrate	Water
			Months	Percent	Percent	Percent	Percent	Percent	Percent
766904..	Texas.....	Jan. 18, 1938	3	9	5	61	12	37	25
		July 26, 1938	9	20	6	15	6	---	---
		Nov. 15, 1938	13	11	10	68	28	83	78
100.....	do.....	Nov. 16, 1938	2	7	3	54	10	55	12
1256.....	do.....	Nov. 18, 1938	3	21	5	56	22	71	41
		Apr. 16, 1938	1 29	4	1	33	4	---	---
272727..	Nebraska.....	July 29, 1938	1 32	6	1	1	0	---	---
		Nov. 10, 1938	1 36	5	1	55	28	71	62
		Feb. 15, 1939	1 39	5	0	61	25	---	---

See footnotes at end of table.



TABLE 6.—*Germination in 49 days of Sporobolus cryptandrus seed of various ages collected in different States—Continued*

Sample No.	State where sample was collected	Date of germination test	Approximate age of seed	Germination, with indicated treatment, at—					
				20° to 35° C.		Room to 35° C.		Room temperature to 35° C. (after prechilling at 3° for 28 days)	
				Potassium nitrate	Water	Potassium nitrate	Water	Potassium nitrate	Water
			Months	Percent	Percent	Percent	Percent	Percent	Percent
769223	Arizona	Sept. 7, 1938	15	16	10	13	7	87	85
		Nov. 9, 1938	17	16	15	33	16	85	89
		Feb. 16, 1939	20	27	15	76	26		
A810	do	Mar. 3, 1939	<sup>1</sup> 14	27	14	59	20	66	50
		May 10, 1935	<sup>2</sup> 7	9	13	54	29	85	63
		Nov. 22, 1935	<sup>2</sup> 13	43	18	70	43	98	73
757544	Wyoming	Mar. 26, 1936	<sup>2</sup> 17	10	4	90	76	97	85
		May 15, 1936	<sup>2</sup> 19	23	8	67	31	76	42
		Sept. 15, 1937	<sup>2</sup> 35	49	21	89	72	88	83
		July 26, 1938	<sup>2</sup> 45	28	16	14	8	56	32
		Nov. 8, 1938	<sup>2</sup> 49	32	22	95	83		
101	Colorado	Nov. 18, 1938	1	9	4	91	69	76	35
		Jan. 18, 1938	5	67	26	92	56	97	86
776903	Oklahoma	July 25, 1938	11	62	19	68	33		
		Nov. 8, 1938	15	61	33	95	56		
O-1914	do	Nov. 21, 1938	2	64	20	99	66	100	77
		Apr. 16, 1938	<sup>1</sup> 18	80	72	90	65	93	74
272728	do	July 29, 1938	<sup>1</sup> 21	65	56	73	54		
		Nov. 14, 1938	<sup>1</sup> 25	63	56	98	85	95	96
		Apr. 16, 1938	<sup>1</sup> 29	64	62	98	95	97	94
272726	Washington	July 28, 1938	<sup>1</sup> 32	82	79	82	73		
		Nov. 8, 1938	<sup>1</sup> 36	70	68	97	98		
		Mar. 7, 1938	1	57	25	96	78	96	97
767694	Utah	July 28, 1938	5	83	45	75	34		
		Nov. 8, 1938	9	85	42	97	83		
		Sept. 12, 1938	1	78	27	87	25	88	67
769232	Kansas	Nov. 9, 1938	2	87	17	92	55	92	84

<sup>1</sup> Actual date of collection not known.<sup>2</sup> Based on assumption that seed was collected in fall of 1934.

## CHANGE WITH AGE

In general, germination was higher for all samples at room temperature to 35° C. alternation than at 20° to 35° whatever the age of the seed when tested (table 6). The beneficial effect of light during germination was observed for seeds up to at least 3 years old. A consistent change of germination with age of the seed was not evident when it was germinated at 20° to 35° alternation. At room temperature to 35° alternation seed germination was very low during the summer months, as the room temperature was more nearly 25° than 20° during this period. Except in these summer tests, there was a general increase of germination at room temperature to 35° alternation as the seed became older up to 4 years, when the experiment was terminated. The nonresistant samples that germinated well at the first test at room temperature to 35° alternation with potassium nitrate showed increased germination with age of seed when moistened with water. With the exception of one sample that gave practically complete germination with water, the use of potassium nitrate increased germination markedly for seed of all ages. A combination of both light and potassium nitrate was essential for maximum germination. Tests that had been prechilled at 3° for 28 days and then placed at room

temperature to 35° alternation tended to show better germination as the seed became older; the difference was most striking when water was used to moisten the substratum.

In the foregoing experiments the tests remained in the germinator for a long period to give full opportunity for all germination possible. However, very little germination occurred after 21 days for either treated or untreated seed, and in some cases there was little germination after 7 or 14 days.

#### SPOROBOLUS FLEXUOSUS

##### GERMINATION OF INTACT SEEDS

The effect of the constant temperatures 20°, 25°, 30°, and 35° C. and of the temperature alternations 15° to 25°, 20° to 30° with light, 10° to 35°, 15° to 35°, 20° to 35°, room temperature to 35°, 20° to 40°, 25° to 40°, and 35° to 10° was determined on the germination of fresh and older seed of *Sporobolus flexuosus* in February 1938. The seed was germinated with potassium nitrate and with water. The seed germinated very poorly and practically in the same proportion at all temperatures, and nitrate and light appeared to have no effect. The highest germination obtained was 15 percent. The results are not given in tabular form.

##### TREATMENT WITH SULFURIC ACID

On November 29, 1938, seed of four samples of *Sporobolus flexuosus*, untreated and treated with approximately 71-percent sulfuric acid for 4 minutes, was germinated at room temperature to 35° C. alternation and at 20° to 35° with the use of potassium nitrate and of tap water. The results of the germination tests are given in table 7. In every case germination of untreated seed was less than half the mean germination of untreated seed and seed treated 4 minutes, indicating a significant effect of acid treatment for 4 minutes. The use of potassium nitrate was not beneficial. Results at the two germination temperatures were similar.

On February 20, 1939, three of these samples of *S. flexuosus*, untreated and treated with sulfuric acid for 4 and 7 minutes, were germinated under the same conditions as in the November test. The results of the germination tests are given in table 8. Maximum germination was obtained in 21 days. A 4-minute treatment was definitely superior to no treatment and to a 7-minute treatment. A 7-minute treatment appeared to injure some of the seeds. A few tests with a 2-minute treatment indicated that a longer period of treatment was necessary. There was no difference in the results at the two germination conditions, indicating that the light received at room temperature was of no benefit. There was an indication of a slight benefit from the use of potassium nitrate.

After transformation of the original data of table 8 to angular values by the use of table 12 of Fisher and Yates (13), an analysis of variance and tests for significance did not indicate any different conclusions from those based on the original values.

The intact seed of the three samples tested in November 1938 and again in February 1939 showed no afterripening effect in 3 months' time.

TABLE 7.—Germination in 56 days of seed of *Sporobolus flexuosus* at two temperature alternations, with and without treatment with approximately 71-percent sulfuric acid, Nov. 29, 1938

Treatment of substratum and sample No.	Observations	Germination after indicated period of acid treatment at—					
		Room temperature to 35° C.			20° to 35° C.		
		0 min-ute	4 min-utes	Mean	0 min-ute	4 min-utes	Mean
Potassium nitrate:	Number	Percent	Percent	Percent	Percent	Percent	Percent
767711.....	2	20.0	63.0	41.50	13.5	58.0	35.75
767741.....	2	5.5	59.5	32.50	6.0	57.5	31.75
767280.....	2	6.0	95.5	50.75	5.0	94.5	49.75
767283.....	2	9.5	88.0	48.75	9.5	80.5	45.00
Total or mean.....	8	10.25	76.50	43.37	8.50	72.62	40.56
Water:							
767711.....	2	11.5	52.5	32.00	7.0	51.0	29.00
767741.....	2	11.5	51.5	31.50	7.0	58.5	32.75
767280.....	2	5.5	94.5	50.00	6.0	94.5	50.25
767283.....	2	6.5	82.0	44.25	9.0	87.0	48.00
Total or mean.....	8	8.75	70.12	39.43	7.25	72.75	40.00
Total or mean.....	16	9.50	73.31	41.40	7.87	72.68	40.28

Treatment of substratum	Total observations	Mean germination for the 2 temperature alternations		
		0 min-ute	4 min-utes	Mean
Potassium nitrate.....	Number	Percent	Percent	Percent
Water.....	16	9.37	74.56	41.96
Potassium nitrate and water.....	16	8.00	71.43	39.71
	32	8.68	72.99	40.84

TABLE 8.—Germination in 21 days of seed of *Sporobolus flexuosus* at two temperature alternations, with and without 71-percent sulfuric acid treatments, Feb. 20, 1939

ORIGINAL DATA FOR ROOM TEMPERATURE TO 35° C.

Treatment of substratum and sample No.	Observations	Germination after acid treatment for—			
		0 min-ute	4 min-utes	7 min-utes	Mean
Potassium nitrate:	Number	Percent	Percent	Percent	Percent
767711.....	2	15.0	84.0	64.0	54.33
767741.....	2	6.0	66.5	66.0	46.16
767283.....	2	3.0	89.5	58.5	50.33
Total or mean.....	6	8.0	80.0	62.83	50.27
Water:					
767711.....	2	9.0	76.0	60.5	48.50
767741.....	2	4.5	60.5	57.5	40.83
767283.....	2	6.0	83.5	51.0	46.83
Total or mean.....	6	6.5	73.33	56.33	45.38
Total or mean.....	12	7.25	76.67	59.58	47.83

TABLE 8.—*Germination in 21 days of seed of Sporobolus flexuosus at two temperature alternations, with and without 71-percent sulfuric acid treatments, Feb. 20, 1939—Continued*

## ORIGINAL DATA FOR 20° TO 35° C.

Treatment of substratum and sample No.	Observations	Germination after acid treatment for—			
		0 min-ute	4 min-utes	7 min-utes	Mean
Potassium nitrate:	Number	Percent	Percent	Percent	Percent
767711 .....	2	8.5	77.5	52.5	46.16
767741 .....	2	7.0	71.0	61.5	46.50
767283 .....	2	7.5	88.5	55.0	50.33
Total or mean .....	6	7.67	79.0	56.33	47.66
Water:					
767711 .....	2	9.0	71.5	45.5	42.00
767741 .....	2	4.5	67.5	51.5	41.17
767283 .....	2	6.0	85.0	39.0	43.33
Total or mean .....	6	6.50	74.66	45.33	42.17
Total or mean .....	12	7.08	76.83	50.83	44.91

## MEAN FOR THE 2 TEMPERATURE ALTERNATIONS

Potassium nitrate .....	12	7.83	79.50	59.58	48.96
Water .....	12	6.50	73.99	50.83	43.77
Water and potassium nitrate .....	24	7.16	76.74	55.20	46.36

## ANALYSIS OF VARIANCE FOR ALL TREATMENTS

Source	D. F.	Mean square	F
Between samples .....	2	132.04	
A (4 v. 7 minutes acid treatment) .....	1	5,598.52	** 58.05
B (4+7 minutes v. (twice) (0) minute acid treatment) .....	1	55,342.56	** 576.96
C (KNO <sub>3</sub> v. H <sub>2</sub> O) .....	1	485.68	* 5.06
D (temperature <sub>1</sub> v. temperature <sub>2</sub> ) .....	1	153.12	1.60
Treatment interactions .....	7	73.02	
Error (sample X treatment) .....	22	95.02	
Between duplicates .....	36	24.93	
Total .....	71		

## ANALYSIS OF VARIANCE FOR 4- AND 7-MINUTE ACID TREATMENTS

Between samples .....	2	146.49	
A (4 v. 7 minutes acid treatment) .....	1	5,598.52	** 41.45
C' (KNO <sub>3</sub> v. H <sub>2</sub> O) .....	1	609.19	4.53
D' (temperature <sub>1</sub> v. temperature <sub>2</sub> ) .....	1	221.02	1.64
Treatment interactions .....	4	77.19	
Error (sample X treatment) .....	14	134.35	
Between duplicates .....	24	36.39	
Total .....	47		

## COMPARISON OF PRECHILLING AND ACID TREATMENTS

In a test begun November 29, 1938, the effect of prechilling the seed at 3° C. for 28 days was determined in comparison with that of a 4-minute sulfuric acid treatment. The pretreated samples were germinated at room temperature to 35° alternation with potassium nitrate and with tap water. The results of the germination tests are given in table 9. The acid pretreatment is better than the prechilling treatment. Only one prechilled sample showed more benefit from the use of potassium nitrate than water.

TABLE 9.—Germination in 56 days of seed of *Sporobolus flexuosus* at room temperature to 35° C. alternation after prechilling or treatment with sulfuric acid Nov. 29, 1938

Sample No.	Observations	Germination with potassium nitrate after indicated treatment			Germination with water after indicated treatment		
		Prechilled 28 days at 3° C.	71-percent H <sub>2</sub> SO <sub>4</sub> for 4 minutes	Mean	Prechilled 28 days at 3° C.	71-percent H <sub>2</sub> SO <sub>4</sub> for 4 minutes	Mean
	Number	Percent	Percent	Percent	Percent	Percent	Percent
767711.....	2	45.5	63.0	54.25	25.0	52.5	38.75
767741.....	2	17.0	59.5	38.25	20.5	51.5	36.00
767280.....	2	21.5	95.5	58.50	18.0	94.5	56.25
727283.....	2	32.0	88.0	60.00	36.5	82.0	59.25
Total or mean ...	8	29.00	76.50	52.75	25.0	70.12	47.56

Treatment of substratum	Total observations	Mean germination for all samples at indicated treatment		Mean
		Germination after prechilling 28 days at 3° C.	Germination after treatment with 71-percent H <sub>2</sub> SO <sub>4</sub> for 4 minutes	
Potassium nitrate and water.....	Number 16	Percent 27.00	Percent 73.31	Percent 50.15

#### SPOROBOLUS CONTRACTUS

##### GERMINATION OF INTACT SEEDS

The effect of constant temperatures of 10°, 15°, 20°, 25°, and 35° C. and of temperature alternations of 15° to 25°, 20° to 30° with light, 10° to 35°, 15° to 35°, 20° to 35°, room temperature to 35°, 20° to 40°, 25° to 40°, and 35° to 10° was determined on the germination of fresh and older seed of *Sporobolus contractus* in February and March 1938. Although all the results were low (0 to 45 percent), they indicated that the higher constant temperatures were better than the lower constant temperatures and that the temperature alternations that included higher temperatures were better than the lower temperature alternations. The use of potassium nitrate was of some benefit at the more favorable temperatures. These results are not given in tabular form.

##### TREATMENT WITH SULFURIC ACID

On November 28, 1938, untreated seed of *Sporobolus contractus* and seed pretreated with approximately 71-percent sulfuric acid for 4 minutes were germinated at room temperature to 35° C. alternation and at 15° to 35°. The effect of potassium nitrate was also included in this study. The results of the germination tests are given in table 10. The results with the 4-minute acid treatment are decidedly better than with no treatment. The 15° to 35° alternation was superior to that of room temperature to 35° for the untreated seed of sample No. 767287, but for the seed treated 4 minutes the difference in tempera-

ture response was not significant. The untreated and treated seeds of sample No. 767287 were benefited by the use of potassium nitrate at both temperatures. The untreated seed of sample No. 767742 germinated very little at either temperature with water or with potassium nitrate.

TABLE 10.—*Germination in 35 days of seed of Sporobolus contractus at two germination temperature alternations, with and without treatment with approximately 71-percent sulfuric acid, Nov. 28, 1938*

Treatment of substratum and sample No.	Observations	Germination after indicated periods of acid treatment at—					
		Room temperature to 35° C.			15° to 35° C.		
		0 minute	4 minutes	Mean	0 minute	4 minutes	Mean
Potassium nitrate:	<i>Number</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>
767287.....	2	46.5	93.0	69.75	69.5	88.5	79.0
767742.....	2	9.5	73.5	41.5	9.5	67.5	38.5
Total or mean.....	4	28.0	83.25	55.62	39.50	78.00	58.75
Water:							
767287.....	2	20.0	81.0	50.5	39.0	82.5	60.75
767742.....	2	7.5	48.5	28.0	10.5	55.0	32.75
Total or mean.....	4	13.75	64.75	39.25	24.75	68.75	46.75
Total or means.....	8	20.87	74.00	47.43	32.12	73.37	52.75

Treatment of substratum	Total observations	Mean germination for the 2 temperature alternations after acid treatment for—		
		0 minutes	4 minutes	Mean
	<i>Number</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>
Potassium nitrate.....	8	33.75	80.62	57.18
Water.....	8	19.25	66.75	43.00
Potassium nitrate and water.....	16	26.50	73.68	50.09

On February 27, 1939, untreated seed of *Sporobolus contractus* and seed treated for 2 and 4 minutes with approximately 71-percent sulfuric acid were germinated in the same manner as described above. The results of the germination tests are given in table 11. The untreated seed gave significantly lower germination than the seed treated for 2 and 4 minutes. The difference between the 2- and 4-minute treatments was not significant. The results with potassium nitrate were not significantly better than the results with water. The significant differences among the samples cannot be attributed entirely to differences in age. Sample No. 767287, which was 20 months old, had afterripened some between the two tests. On the other hand, sample No. A8763, 1-month-old seed, showed a higher germination than the 12-month-old seed of sample No. 767742. All three samples required acid treatment before maximum germination could be obtained.

TABLE 11.—Germination in 56 days of seed of *Sporobolus contractus* at room temperature to 35° C. alternation after treatment with approximately 71-percent sulfuric acid for various periods, Feb. 27, 1939

## ORIGINAL DATA

Treatment of substratum and sample No.	Observations	Germination after acid treatment for—			Total observations	Mean germination
		0 minute	2 minutes	4 minutes		
Potassium nitrate:	Number	Percent	Percent	Percent	Number	Percent
767287	2	74.0	90.0	85.5	6	83.16
767742	2	11.0	68.5	73.5	6	51.00
A8763	2	51.5	97.0	85.0	6	77.83
Total or mean	6	45.50	85.16	81.33	18	70.66
Water:						
767287	2	51.5	84.5	61.5	6	65.83
767742	2	6.0	53.5	66.5	6	42.00
A8763	2	38.5	95.0	72.5	6	68.66
Total or mean	6	32.00	77.60	66.83	18	58.83
Potassium nitrate and water, mean	12	38.75	81.41	74.08	36	64.74

## ANALYSIS OF VARIANCE FOR ALL TREATMENTS

Source	D. F.	Mean square	F
Between samples	2	3,002.25	
A (2 minutes v. 4 minutes acid treatment)	1	322.67	
B (2+4 minutes v. twice (0) minute acid treatment)	1	12,168.00	**36.65
C (KNO <sub>3</sub> v. H <sub>2</sub> O)	1	1,260.25	3.80
Treatment interactions	2	43.00	
Error (sample X treatment)	10	331.98	
Between duplicates	18	22.75	
Total	35		

## ANALYSIS OF VARIANCE FOR 2- AND 4-MINUTE ACID TREATMENTS

Between samples	2	998.37	
A (2 v. 4 minutes acid treatment)	1	322.67	1.84
C' (KNO <sub>3</sub> v. H <sub>2</sub> O)	1	726.00	4.13
Treatment interaction	1	73.50	
Error (sample X treatments)	6	175.00	
Between duplicates	12	12.50	
Total	23		

## COMPARISON OF PRECHILLING AND ACID TREATMENTS

In a test begun November 28, 1938, the effect of prechilling at 3° C. was determined on the seed of *Sporobolus contractus*. The results of the prechilling tests and, for comparison, the results with seed pretreated with approximately 71-percent sulfuric acid for 4 minutes are given in table 12. The difference between the two treatments is not significant for sample No. 767287, but for sample No. 767742 the acid pretreatment is superior to the prechilling treatment. The test with potassium nitrate is significantly better than that with water except that germination of sample No. 767287 was practically complete either with potassium nitrate or with water when the seed had been prechilled.

TABLE 12.—*Germination in 56 days of seed of Sporobolus contractus at room temperature to 35° C. alternation after prechilling or treatment with approximately 71-percent sulfuric acid, Nov. 28, 1938*

Treatment of substratum and sample No.	Observations for each treatment	Germination after indicated treatment		Total observations	Mean germination
		Prechilled 28 days at 3° C.	Treated with approximately 71-percent H <sub>2</sub> SO <sub>4</sub> for 4 minutes		
Potassium nitrate:	<i>Number</i>	<i>Percent</i>	<i>Percent</i>	<i>Number</i>	<i>Percent</i>
767287	2	91.5	93.0	4	92.25
767742	2	53.5	73.5	4	63.50
Total or mean	4	72.50	83.25	8	77.87
Water:					
767287	2	94.0	81.0	4	87.50
767742	2	23.0	48.5	4	35.75
Total or mean	4	58.50	64.75	8	61.62
Total or mean for both treatments	8	65.50	74.00	16	69.75

#### SPOROBOLOUS GIGANTEUS

#### GERMINATION OF INTACT SEEDS

The effect of the constant temperatures of 20°, 25°, 30°, and 35° C., of the temperature alternations of 15° to 25°, 20° to 30° with light, 10° to 35°, 20° to 35°, room temperature to 35°, 20° to 40°, 25° to 40°, and 35° to 10°, and of prechilling at 3° for various periods was determined on the germination of fresh and old seed of *Sporobolus giganteus*. The germination in any individual constant- or alternating-temperature test was less than 20 percent. The use of potassium nitrate and of light appeared to have no effect. The percentage of germination could be raised by prechilling for 28 to 56 days, but maximum germination was not obtained. These results are not given in tabular form.

#### TREATMENT WITH SULFURIC ACID

Seed of *Sporobolus giganteus* was treated with approximately 71-percent sulfuric acid for 5, 7, and 9 minutes and germinated along with untreated seed at room temperature to 35° C. alternation with the use of potassium nitrate and with water. The germination results are given in table 13. The tests were continued for 49 days, but there was no additional germination after the twenty-first day. The treatment for 9 minutes was superior to the other treatments for sample No. 767281, whereas the results with sample No. A8392 showed the 5- and 7-minute treatments to be superior. If the means of the two samples are considered, germination of the seed treated 7 and 9 minutes is better than that of the untreated seed or the seed treated 5 minutes. The difference between the test with potassium nitrate and that with water was significant for the different lengths of acid treatment for sample No. 767281, but was not significant for the other sample.



TABLE 13.—*Germination in 21 days of seed of Sporobolus giganteus at room temperature to 35° C. alternation after treatment with approximately 71-percent sulfuric acid for various periods, February 1939*

Treatment of substratum and sample No.	Observations for each treatment	Germination after treatment for—				Total observations	Mean germination
		0 minute	5 minutes	7 minutes	9 minutes		
Potassium nitrate:	<i>Number</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Percent</i>	<i>Number</i>	<i>Percent</i>
767281.....	2	4.5	45.0	60.0	72.5	8	45.50
A8392.....	2	19.5	71.0	76.5	61.5	8	57.12
Total or mean.....	4	12.00	58.00	68.25	67.00	16	51.31
Water:							
767281.....	2	1.0	16.0	43.5	49.5	8	27.50
A8392.....	2	19.0	73.5	73.5	59.0	8	56.25
Total or mean.....	4	10.00	44.75	58.50	54.25	16	41.87
Total or mean for both treatments.....	8	11.0	51.37	63.37	60.62	32	46.59

## CHANGE WITH AGE

Sample No. 767281, tested in February 1938 and again in February 1939, showed no afterripening.

## GERMINATION OF INTACT SEEDS OF SPOROBOLUS AIROIDES

The results of germination tests under different conditions with four samples of seed of *Sporobolus airoides* are given in table 14. The two older samples, Nos. 272724 and A8501, germinated readily and equally well at the high alternating temperatures without prechilling. Of the two samples having comparatively fresh seed, one was benefited by prechilling and the other was benefited slightly by the use of potassium nitrate. Although a high percentage of germination was not obtained with sample No. 767700, the results with the prechilled seed apparently represented the full value of the sample, for the seed germinated promptly or decayed.

TABLE 14.—*Germination in 28 to 35 days of seed of Sporobolus airoides at two temperature alternations after various treatments*

Sample No.	Date of germination test	Temperature of germination chamber (°C.)	Seed prechilled at 3° C.	Germination on substratum moistened with—	
				Potassium nitrate	Water
			<i>Days</i>	<i>Percent</i>	<i>Percent</i>
272724.....	May 18, 1938	Room to 35.....	0	89	94
		do.....	7	89	87
		do.....	14	95	91
		do.....	21	92	93
		do.....	0	37	30
767700.....	Mar. 8, 1938	20 to 35.....	0	43	43
		do.....	14	64	68
		do.....	21	67	66
		do.....	28	57	63
		do.....	0	97	81
767716.....	Mar. 29, 1938	Room to 25.....	0	86	84
		do.....	28	83	87
		do.....	0	96	95
A8501.....	Feb. 28, 1939	do.....	0	94	95
		20 to 35.....	0		

GERMINATION OF INTACT SEEDS OF SPOROBOLUS WRIGHTII

The seed of *Sporobolus wrightii* was germinated at four temperatures with potassium nitrate and with water and with various prechilling treatments. The results are given in table 15. The seed germinated readily at a warm alternating temperature; it did not appear to be nitrate-sensitive at a favorable temperature. Sample No. A666 appeared to be somewhat light-sensitive.

TABLE 15.—*Germination in 35 days of seed of Sporobolus wrightii at various temperatures*

Sample No.	Date of germination test	Approximate age of seed	Germination temperature alternation (°C.)	Prechilled at 3° C.	Germination on substratum moistened with—	
					Potassium nitrate	Water
		Months		Days	Percent	Percent
769224	Sept. 13, 1938	10	20 to 30	0	81	49
			20 to 35	0	96	87
			Room to 35	0	91	91
			20 to 40	0	88	90
			20 to 35	14	92	95
			Room to 35	14	99	93
			20 to 35	28	94	95
			Room to 35	28	97	95
A666	Feb. 28, 1939	6	20 to 35	0	76	65
			Room to 35	0	86	80

GERMINATION OF INTACT SEEDS OF SPOROBOLUS ASPER

Seed of *Sporobolus asper* was germinated at three temperature alternations with potassium nitrate and with water and with prechilling treatment. The results of the tests are given in table 16. Comparatively fresh seed appeared to require prechilling for 14 days and the use of potassium nitrate for maximum germination. Seven months later the need of prechilling and nitrate had disappeared as the seed germinated readily with tap water at room temperature to 35° C. alternation, at 20° to 35°, and at 15° to 35°.

TABLE 16.—*Germination in 49 days of seed of Sporobolus asper (sample 272725, from Woodward, Okla., 1937) at different temperature alternations*

Date of germination test and treatment of substratum	Germination at indicated temperature alternations after prechilling at 3° C. for various periods				
	Room to 35° C.			20° to 35° C.	15° to 35° C.
	0 days	14 days	35 days	0 days	0 days
April 16, 1938:	Percent	Percent	Percent	Percent	Percent
Potassium nitrate	39	94	88	68	
Water	17	71	70	54	
Nov. 28, 1938:					
Potassium nitrate	96	93		94	96
Water	90	96		93	96

WATER ABSORPTION BY SEED OF SPOROBOLUS

Water-absorption tests were made to determine whether the effect of the coats in restricting germination of seed of *Sporobolus* spp. was

due to limiting of water absorption, as has been suggested by Jackson (23) and by Wilson (38).

The pericarp of all species studied became gelatinous in contact with water and, with the exception of *Sporobolus airoides* and *S. wrightii*, appeared to split and so release the seed. With *S. airoides* and *S. wrightii* the pericarp became gelatinous in contact with water but remained around the seed. Because the pericarp did not seem to be concerned in the coat restriction of germination, it was removed from the untreated seeds to make them comparable with the seeds from which the pericarp had been removed by acid treatment. The seeds were placed on moist blotting paper, and the pericarp was removed, under a lens, from 200 seeds of one or more samples of each species. Preliminary tests indicated that no appreciable amount of water was absorbed by the seed during removal of the pericarp. The acid-treated seeds and the nontreated seeds with pericarp removed were weighed, soaked in water for the periods indicated in table 17, and reweighed.

TABLE 17.--Water absorption by seed of *Sporobolus* species

Species	Resistance and treatment	Samples averaged	Average weight of 200 seeds				After 48 hours' soaking	Increase
			Dry	With-out pericarp	After 24 hours' soaking	Increase		
		Number	Gram	Gram	Gram	Percent	Gram	Percent
<i>S. cryptandrus</i>	Resistant	5	0.0182	0.0170	0.0208	22.29	0.0222	125.92
	Nonresistant	8	.0171	.0160	.0195	22.23		
	Acid-treated	5		.0174	.0219	126.11		
<i>S. flexuosus</i>	Resistant (a)	3	.0220	.0193	.0242	25.00	.0274	142.20
	Resistant (b)	2	.0243	.0212	.0258	22.19	.0275	130.02
	Acid-treated (a)	3		.0194	.0262	134.34		
<i>S. contractus</i>	Acid-treated (b)	2		.0215	.0279	129.57		
	Resistant	4	.0208	.0238	.0296	24.20	.0313	136.83
	Acid-treated <sup>1</sup>	4		.0246	.0314	27.54	.0346	140.96
<i>S. giganteus</i>	Resistant	1	.0535	.0478	.0587	22.80	.0635	132.84
	Acid-treated	1		.0464	.0621	133.83		
<i>S. airoides</i>	Nonresistant	4	.0468	.0426	.0602	42.45		
<i>S. wrightii</i>	Nonresistant	2	.0492	.0440	.0610	37.70		
<i>S. asper</i>	Nonresistant	1	.1542	.1437	.1787	24.35	.1877	130.62
	Acid-treated	1		.1475	.1909	29.42	.1972	133.69

<sup>1</sup> Few to many seed showing evidence of germination.

<sup>2</sup> Average increase in weight after 7½ hours' soaking, 24.17 percent.

The very first evidence of germination is indicated in table 17, since it introduced another factor that greatly increased water absorption. The seed coats of none of the species studied excluded water. In *Sporobolus cryptandrus* the untreated seed of resistant and of nonresistant samples absorbed practically the same amount of water and at approximately the same rate. In *S. flexuosus* the samples labeled "resistant a" germinated better after acid treatment than those samples indicated as "resistant b"; however, there was little difference in the rate of water absorption of the two groups. The acid-treated seeds of *S. contractus* and of *S. giganteus* probably absorb water at a faster rate than the nontreated seeds. The nonresistant seeds of *S. airoides* and *S. wrightii* appear to absorb water at a faster rate than the other species. However, an error is introduced by the early germination of the acid-treated and nonresistant lots.

Although there was an indication of some increase in rate of water absorption by acid-treated and by nonresistant samples, it would not appear from these results that restriction of water absorption by the coats of *Sporobolus* seed was a cause of resistance to germination.

## DISCUSSION

Much has been published on the need of light, nitrate, and special temperatures for the germination of seeds. Of the seven species of *Sporobolus* studied, *Sporobolus airoides*, *S. wrightii*, *S. asper*, and samples of *S. cryptandrus* from Colorado, Oklahoma, Washington, Utah, and Kansas germinated readily at a suitable temperature alternation, although in some cases the addition of light and potassium nitrate was necessary for maximum germination. On the other hand, *S. flexuosus*, *S. contractus*, *S. giganteus*, and samples of *S. cryptandrus* from Texas, Arizona, Nebraska, and Wyoming gave very low germination at a wide range of constant and alternating temperatures, and nitrate and light had little or no effect. Further treatment was necessary for best germination. According to Hitchcock (21), *S. flexuosus*, *S. contractus*, *S. giganteus*, and *S. cryptandrus* are closely related.

Harrington (19) and others have published on the effect of the alternation of temperatures on germination; and Crocker (8), Gassner (15), Shull (29), and Harrington (19) have pointed out the beneficial effect of high germination temperatures on intact seeds restricted in their germination by the seed coat or glumes. *Sporobolus cryptandrus* seed germinated best at a high alternating temperature and there was an indication that *S. contractus* was benefited by high temperatures, although the results with untreated seed were poor at all temperatures. In the absence of special treatment, *S. flexuosus* and *S. giganteus* germinated poorly at both high and low temperatures.

The seed of resistant samples germinated readily after treatment with approximately 71-percent sulfuric acid when supplied with the optimum conditions for germination. Concentrated sulfuric acid has long been used for overcoming the impermeability to water of hard-seeded legumes, but Hiltner (20), in 1910, was the first to apply sulfuric acid treatment to grass seed. Since then several workers have used concentrated sulfuric acid successfully on grass seed: Harrington (17, 18) on Johnson grass and wheat, Morinaga (26) and Byran (6) on *Cynodon dactylon*, Burton (7) on *Paspalum notatum* and *P. dilatatum*, and Huntamer<sup>4</sup> and Stoddart and Wilkinson (32) on *Oryzopsis hymenoides*. Burton (7) found crude sulfuric acid of approximately 75-percent strength to be very effective. The writer (34, 35, 36), has found approximately 71-percent sulfuric acid very effective in overcoming the coat resistance in seed of *Danthonia spicata* and of *Oryzopsis hymenoides* and partially effective for *Eragrostis brizantha*. Since the resistance to germination is overcome by treatment with acid, it may be assumed that delayed germination in *Sporobolus flexuosus*, *S. contractus*, *S. giganteus*, and some samples of *S. cryptandrus* is due to some influence of the seed coat. The gelatinous pericarp apparently is not related to resistance to germination, as the species that germinated readily included both those

<sup>4</sup> HUNTAMER, MAY Z. DORMANCY AND DELAYED GERMINATION OF ORYZOPSIS HYMENOIDES. State College of Washington. [Unpublished thesis.]

in which the seed is released from the gelatinous pericarp and those in which the seed is retained within the pericarp.

Davis and Rose (12) were the first to point out the beneficial effect of stratification at 5° C. for overcoming the deep-seated embryo dormancy of *Crataegus*. Toole (33) and Whitcomb (37) used a short prechilling treatment at 5° to overcome the dormancy of freshly harvested wheat, which is due to the restriction of the coats as has been pointed out by Harrington (18). For the resistant *Sporobolus* species, prechilling at 3° for 2 to 4 weeks brought about maximum germination with some samples, but with most samples treatment with sulfuric acid was superior. The nonresistant samples were benefited by prechilling only when the seed was fresh.

Gassner (15), Magnus (24), and Gardner (14) found for various kinds of seeds that the removal of the coat or glumes eliminated the need of special germination conditions. Morinaga (26) found a similar condition for *Typha latifolia* and *Cynodon dactylon*, but found that breaking the seed coats of *Poa compressa* and of *Apium graveolens* did not do away with the need of alternating temperatures and other special factors. Böhmer (5) found that the effect of light on the light-inhibited seeds of *Nigella sativa* was the same after removal of the coat. Removal of the lemma and the palea was found by Andersen (1) for *Poa compressa* and by Maier (25) for *Phleum pratense* not to change the requirement for light for maximum germination, although Andersen found that seed of *P. compressa* would germinate without the use of potassium nitrate after the removal of the glumes. The writer found that seed of *Sporobolus cryptandrus* was still benefited by light and nitrate after modification of the coat by acid treatment or prechilling. *S. contractus* and *S. flexuosus* showed little or no effect of light or nitrate either before or after acid treatment.

The slight dormancy that may be encountered in fresh seed of *Sporobolus airoides*, *S. asper*, and nonresistant samples of *S. cryptandrus* is of short duration. The resistant samples of *S. cryptandrus* show some evidence of afterripening and there is a slight change with age in germination of seed of *S. contractus*, but even after a year or more of storage additional treatment was required for complete germination. There was no evidence that the germination response of *S. flexuosus* and *S. giganteus* changed with age of the seed.

The germination of the species of *Sporobolus* studied is rapid if all requirements for germination are supplied. Nonresistant species have completed their germination in 14 to 21 days. An occasional sample required an added 7 to 14 days for a few remaining seeds to germinate. The small amount of germination that occurs in those samples in which germination is restricted by the coats takes place quickly; the remaining seeds then remain indefinitely in a dormant condition. When these resistant samples have been treated with acid, a large portion of the germination takes place in 2 days and germination is completed in 14 to 21 days.

Crocker (8, 10) has emphasized the fact that the delayed germination in seeds of many plants often may be due to the seed coats rather than to the embryos. Nobbe (27), Nobbe and Haenlein (28), Crocker (8), and others have reported on seeds having coats entirely impermeable to water; and Crocker (8, 9) has reported on seeds having coats that limit the supply of water necessary for germination. In seed of *Alisma plantago*, Crocker and Davis (11) have shown that the

seed coat causes mechanical restraint. That enclosing structures interfere with oxygen absorption by the embryo and perhaps with carbon dioxide elimination as well has been shown by Crocker (8), Shull (29, 30), Becker (3), Gassner (15), and Atwood (2). Contrary to the belief of Jackson (23) and of Wilson (38), the seed coats of *Sporobolus* do not prevent water absorption, and a comparison of the rate of absorption by the seed of nonresistant with that by the seed of resistant samples or a comparison of nontreated and treated resistant samples would indicate that limitation of water supply is not the cause of delayed germination. It is believed that the coats might restrict gas exchange, but the writer has not worked on this phase of the problem.

### SUMMARY AND CONCLUSIONS

A study was made of the optimum conditions for the germination of seven species of *Sporobolus*, of the variability of germinating power within the species, and of some special treatments for overcoming the resistance of some species to germination.

Germination percentage for *Sporobolus cryptandrus* was low at all constant temperatures, but high constant temperatures were more effective than the lower ones. An alternation of room temperature to 35° C. with light and potassium nitrate gave complete germination of the viable seeds of some samples. Other samples germinated best at these conditions, but maximum germination was not obtained. These resistant samples responded to pretreatment with 71-percent sulfuric acid. A 2-minute acid treatment was sufficient for most samples, but a few samples required 5- to 7-minute treatments. A 9-minute treatment was injurious in all cases. The treated seed responded best to the same conditions for germination as did the untreated seed. Prechilling for 4 to 8 weeks was beneficial and in general as effective as acid treatment. Small seeds removed by a 0.508-mm. sieve were of low viability. Resistant samples did not come from any one geographical region. There is evidence of afterripening; that is, seed of originally resistant samples tended to germinate better after aging.

*Sporobolus flexuosus* and *S. giganteus* germinated poorly and with little variation over a wide range of constant and alternating temperatures. *S. contractus* also germinated poorly at all temperatures, but there was an indication that higher constant temperatures and the temperature alternations including the higher temperatures were superior to the lower constant and alternating temperatures. The use of potassium nitrate and light had no effect on intact seed of *S. flexuosus* or *S. giganteus*, but for *S. contractus* there was some benefit from the use of potassium nitrate at the more favorable temperature. All three species responded to treatment with 71-percent sulfuric acid. Such treatment for 4, 2 or 4, and 7 to 9 minutes appeared to be sufficient for *S. flexuosus*, *S. contractus*, and *S. giganteus*, respectively. After acid treatment the seed of the above three species germinated well at room temperature to 35° C. alternation. Seed of *S. flexuosus* and of *S. giganteus* showed no evidence of afterripening after 3 to 12 months of storage. Seed of *S. contractus* 1 to 1½ years old varied in its afterripening response but still required acid treatment for maximum germination. Prechilling was not so good as acid treatment for *S. flexuosus*.

Fully afterripened seed of *Sporobolus airoides*, *S. wrightii*, and *S. asper* germinated promptly at alternation of 20° to 35° C. or of room temperature to 35°. There was an indication that fresh seed of *Sporobolus airoides* required either nitrate or prechilling and that *S. asper* required prechilling and nitrate for maximum germination. Seed of *S. wrightii* was not sensitive to potassium nitrate at a favorable temperature; one sample appeared to be somewhat light-sensitive.

The seed coat of the species of *Sporobolus* studied does not prevent the absorption of water.

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# DIFFERENTIATION OF PHYSIOLOGIC RACES OF UROMYCES PHASEOLI TYPICA ON BEAN<sup>1</sup>

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## INTRODUCTION

Two physiologic races of the bean rust organism (*Uromyces phaseoli typica* Arthur), one collected in southern California and the other in the vicinity of Washington, D. C., were differentiated in 1935 by Harter, Andrus, and Zaumeyer (3).<sup>3</sup> The one from California was designated form 1, and the one from the East, form 2. More recently Dundas and Scott (1), Parris,<sup>4</sup> Harter (2), and others have recognized the existence of additional physiologic races and have utilized this information in the production of bean varieties (*Phaseolus vulgaris* L.) resistant to rust.

Since the discovery of forms 1 and 2, the existence of many physiologic races has been proved from bean rust material collected from various parts of the country.

The different races described in this paper were differentiated on the basis of their parasitic action on seven varieties of beans used as differential hosts. (See table 1.)

## GEOGRAPHIC DISTRIBUTION

Bean rust seems to be limited in its distribution to those regions where the relative humidity is at least 95 percent for 8 to 10 hours at a time when viable conidia are present. This requirement apparently is not met in most of the States west of the Rocky Mountains with the exception of isolated regions along the Pacific coast, nor in parts of the Great Plains. East of the Rocky Mountains, it may or may not occur, depending, it is believed, on whether or not sufficient humidity prevails. In the more humid parts of the United States, such as in many of the Southern, Eastern, and New England States, rust occurs in varying degrees almost every year. Inasmuch as many varieties are commercially resistant to several of the physiologic races, those that are grown in certain localities often determine, in part at least, the occurrence of rust infection.

The extent of infection, and consequently the amount of loss that occurs locally, depends on several factors, such as the proper atmospheric moisture and suitable temperature concomitant with the presence of viable conidia. That such a combination of conditions does not always occur is evidenced by the fact that rust may not appear more than once or twice during a period of 10 years. The lack of suitable atmospheric humidity is probably the most important governing factor where rusts

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<sup>3</sup> Italic numbers in parentheses refer to Literature Cited, p. 731.

<sup>4</sup> PARRIS, G. K. THE REACTIONS OF INTRODUCED BEAN VARIETIES TO RUST (*UROMYCES PHASEOLI TYPICA*) IN HAWAII. U. S. Dept. Agr. Bur. Plant Indus., Plant Dis. Rptr. 22: 424-428. 1938. [Mimeographed.]

occur only occasionally. Rust spores are probably present every year. Stakman et al. (6) have shown that the spores of wheat rust are carried long distances by the wind; this is probably true also of the spores of bean rust.

Townsend (8) stated that teliospores of the bean rust were not produced in Florida and that under a variety of temperature and moisture conditions most of the urediospores had lost their viability after 14 days and none were viable after 3 months in storage, a length of time not sufficient to carry the rust from the spring to the fall or winter crop in Florida. Parris <sup>5</sup> also stated that the urediospore was the only type found in the Hawaiian Islands. Townsend (8) attributes the frequent occurrence of the rust in Florida to the transport of the spores by wind currents from infected plants in regions to the north. These facts would suggest that, if suitable environmental conditions prevail, a rust epidemic might appear in any given region though no rust had occurred there for many years.

#### MATERIALS AND METHODS

Bean rust was collected at various places in the United States and Hawaii by the writers, or sent to them by workers in the various colleges and experiment stations. Since the rust does not develop normally in hot weather in a greenhouse, any rust material that was received in June, July, or August was placed in cold storage and held until the weather was cool enough to justify making inoculations. If the quantity of material was sufficient and the weather conditions proved favorable for infection, the differential varieties were inoculated. If the amount of spore material was insufficient for the inoculation of an entire set of differentials, it was increased by the inoculation of a few plants of a susceptible variety, usually the Pinto, from which spores were taken later to inoculate a set of differential varieties. An attempt was made to keep spore material of each physiologic race available from the time it was first collected. This could be accomplished (1) by inoculating susceptible plants at frequent intervals during the year or (2) by the storage of infected leaves. The urediospores do not retain their viability for a very long period of time, and it frequently happened that they had lost their viability or survived in so limited a number that they had to be increased before large-scale inoculations could be attempted. Poor results were obtained from inoculations made during the summer under greenhouse conditions, and outdoor inoculations increased the danger of mixing the different physiologic races.

An ordinary electric refrigerator partly solved the problem of prolonging the life of the rust spores, but even under such conditions of storage the percentage of survivals was usually small at the end of 3 or 4 months. Some of the fatalities were due to molds that parasitized the spores even in a refrigerator and destroyed their viability. Drying the leaves from 2 to 4 days in the laboratory at room temperature between layers of some absorbent paper partially eliminated the danger from molds. Some experiments on methods for lengthening the time the urediospores could be maintained in a viable condition showed that there was some correlation between temperature and longevity. From these experiments it developed that if the spores

<sup>5</sup> See footnote 4.

were stored in the ice compartment of a refrigerator they remained viable for many months, and good infection was obtained from urediospores stored for more than 2 years in a refrigerator cooled to  $-20^{\circ}\text{C}$ .

After the adoption of the cold-storage method for maintaining viability of rust material, it was possible to have viable spores available at all times. Viability apparently was maintained for a longer period on leaves bearing the rust, although good results were obtained by storing the spores in the ice compartment of a refrigerator in small vials. While the spores stored in vials apparently did not remain viable as long as those in contact with the leaves, such storage was a convenient method and required only a small amount of space.

Pure lines of the different physiologic races were difficult to maintain. A single field collection of rust often consisted of two or three distinct physiologic races, although in practically every case one form greatly predominated, often as much as 5 to 10 or more to 1. The urediospores predominated mostly in the greenhouse, although teliospores may have developed under certain conditions on moribund leaves in about 3 weeks after inoculation. The urediospores escaped easily from the sorus and floated about in the air currents, which brought about a considerable amount of contamination. Experiments have demonstrated that urediospores will not germinate on the leaves in the open greenhouse during the winter months when artificial heat is required. On the other hand the spores remained viable for at least 15 days, and good infection was obtained when the plants were confined in an infection chamber for 24 hours. Moistening the plants before they were placed in the infection chamber was not necessary. All possible care was taken to isolate the differential varieties or other inoculated plants by placing them in different compartments and greenhouses. In spite of these precautions a certain amount of contamination resulted which sometimes confused the results. Greenhouse attendants and others engaged in watering, handling, and inspecting the plants may have transferred the spores to other inoculated or uninoculated plants. While care was taken to control insects, some were generally present which must be looked upon as agents in the dissemination of the rust spores. When the results were rendered uncertain by mixtures, the rust was pure-lined by starting with urediospores taken from a single sorus. This procedure greatly reduced the number of alien spores, but it was no guarantee of absolute purity, owing either to the presence of foreign spores on the leaf to which the spores of a single sorus were transferred or to contaminants in the sorus itself.

The experiments were conducted in the greenhouse. Conditions were not ideal but were preferable to those outdoors, where there were no barriers to the general mixing of the different races. The results from greenhouse inoculations may not be identical with those obtained under field conditions; nevertheless no considerable amount of deviation was observed, and it is believed that the data can be accepted as sufficiently dependable for all practical needs. Most of the inoculations were made between September and May. During this period environmental conditions varied greatly. In the fall and spring vigorous host plants were easily produced, but during the winter months, when there are many cloudy days, the plants sometimes lacked vigor and the rust did not develop quite as copiously and

uniformly as it did when the days were longer and there was more sunshine. The lack of uniformity of environmental conditions during the different seasons of the year accounted in part at least, for variations in amount and degree of infection of some of the differentials when duplicated at different times. These variations have been a source of confusion in interpreting the results that were obtained at different times on some of the differentials, especially those varieties that showed a certain degree of resistance.

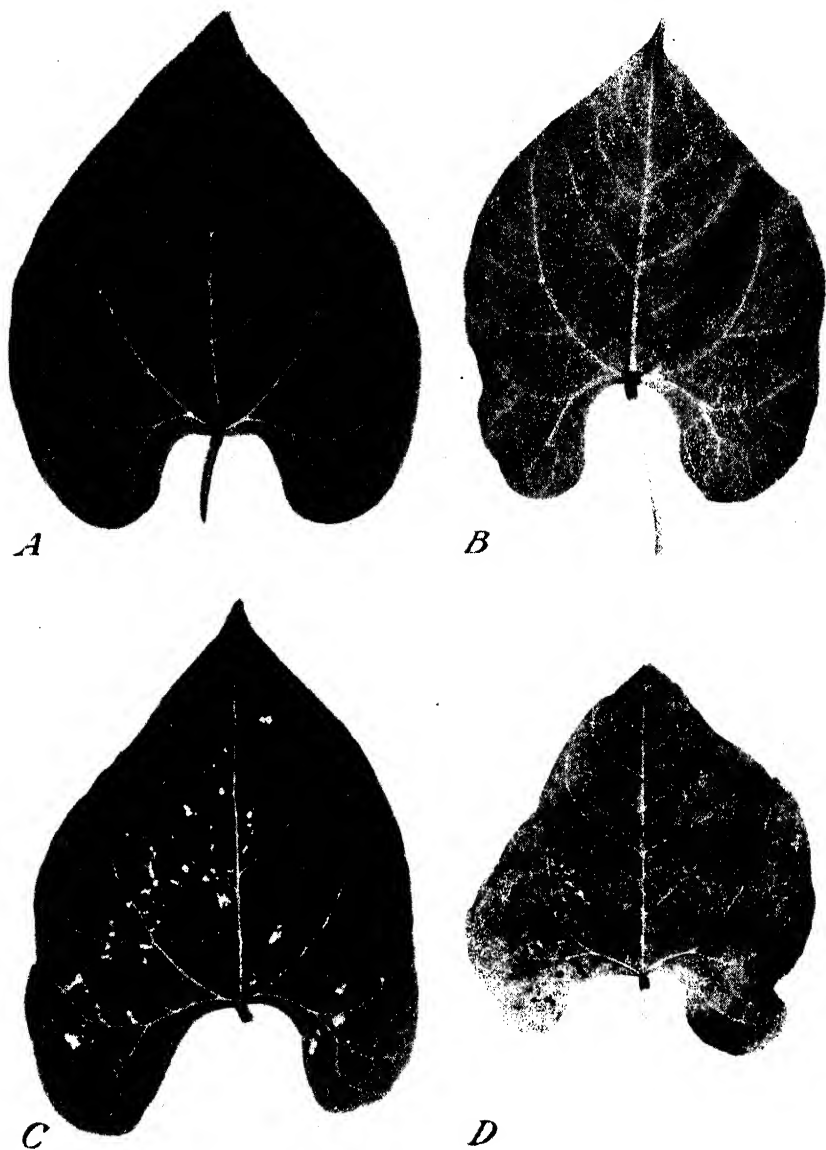
The greatest variations in results occurred mostly in the winter months, when the days were short, the light intensity reduced, and the temperature sometimes too low for the normal growth of the plants. Under such conditions, plants were often yellowed and the sori developed slowly. The percentage of infections was usually low, and mesothetic reactions occurred on certain varieties. Harter, Andrus, and Zaumeyer (3) found that the number of pustules on plants confined in an infection chamber in subdued light decreased as the length of time in the infection chamber beyond 48 hours increased. The decrease in the amount of infection was attributed largely to a lowering of the functional activity of the plant in the absence of sufficient light to support normal growth. Wei (9) stated in this connection that temperatures ranging from 16° to 28° C. did not change the type of reaction, but in some cases both the high and low temperatures increased the proportions of the X type<sup>6</sup> of infection. Low temperatures increased the incubation period.

The seven differential varieties of beans employed in identifying the various physiologic races considered here were as follows: (1) U. S. No. 3, a white-seeded Kentucky Wonder type; (2) Bountiful (No. 181),<sup>7</sup> a common garden bean variety of the bush type; (3) a strain of California Small White (No. 643), a field type grown principally in California; (4) a strain of Pinto (No. 650), a speckled field bean grown extensively in the intermountain region of the West; (5) a selection (No. 765) from the Kentucky Wonder Wax variety; (6) a medium-late white-seeded Kentucky Wonder hybrid (No. 780); (7) a brown-seeded Kentucky Wonder hybrid (No. 814). Seed of each of the differential varieties originated from a single plant selection and was increased each year in Colorado. Occasionally an off-type plant appeared in some of the varieties, which doubtless resulted from an occasional natural field cross with some other variety. Off-type plants were removed so that only rarely was there any variation in the reaction of any of the plants of a single differential. The different rust collections were identified from the reaction on the seven differential hosts described above.

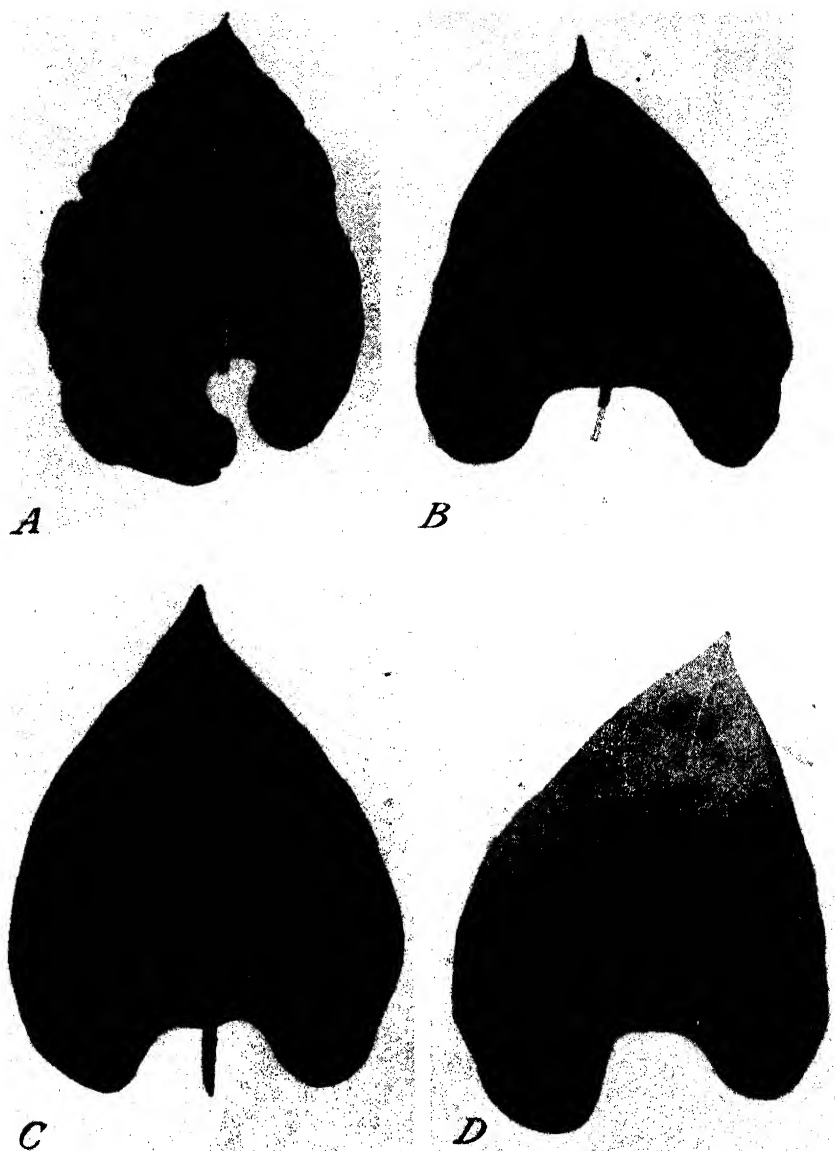
Inoculations were made by brushing spores suspended in water on the upper or lower surface of the primary leaves by means of a camel's-hair brush when the leaves were about one-half to two-thirds grown. Just as good infection was obtained by dusting the spores on the dry leaves. Since the simple leaves matured 7 to 10 days before the trifoliate leaves were fully grown, they were preferred for inoculation purposes. Identical results were obtained with both types of leaves if all other conditions were alike. Young leaves were preferred. Experiments showed that if old simple leaves were inoculated infection took place but the sorus did not attain its maximum

<sup>6</sup> Used as the equivalent of mesothetic.

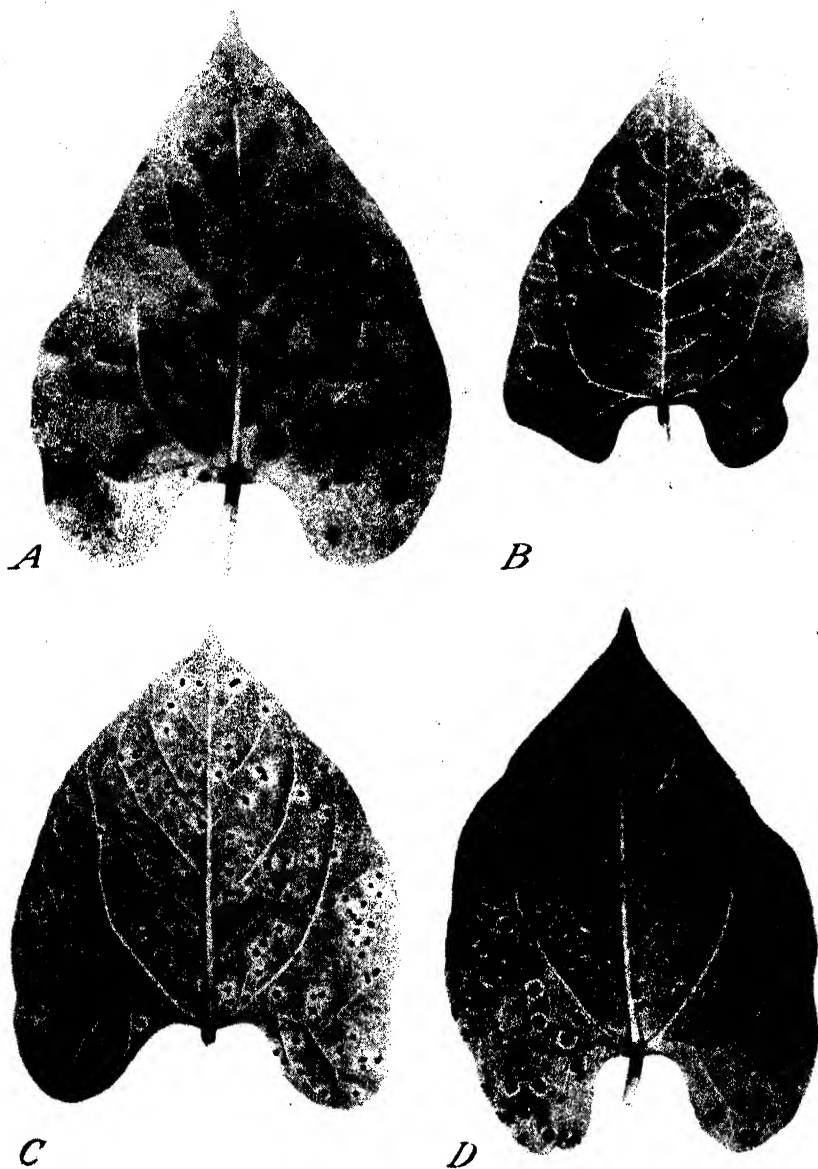
<sup>7</sup> The numbers in parentheses following varietal designations are pure-line selections carried under these numbers in the seed files of the writers.



Types of infection, grades 0, 1, and 2, produced by several races of rust on different varieties of beans. *A*, Grade 0 infection. Brown-seeded Kentucky Wonder hybrid (No. 814) inoculated with rust race 2, showing the highest degree of resistance. *B*, Grade 1 infection. The necrotic lesions are small and more or less round in outline, showing a high degree of resistance. No spores in the sori. Kentucky Wonder Wax (No. 765) inoculated with race 10. *C*, Grade 1 infection. Note the difference in the type of lesions from those shown in *B*, Kentucky Wonder Wax (No. 765) inoculated with race 1. *D*, Grade 2 infection. The lesions differ in type from those shown in *B* and *C*. A few spores occur in some of the sori. U. S. No. 3 inoculated with rust race 1.



Types of infection, grades 2, 3, and 4, produced by several races of rust on different varieties of beans. A, Grade 2 infection, showing a different type of lesion from those shown in plate 1, B, C, and D. Pinto variety inoculated with rust race 10. B, Grade 3 infection. Note difference in the type of lesions from those in A. White-seeded Kentucky Wonder hybrid (No. 780) inoculated with rust race 12. C, Grade 3 infection. A different type of lesion from those shown in A and B. There is very little necrotic tissue outside the sori. White-seeded Kentucky Wonder hybrid (No. 780)  $\times$  Pinto inoculated with race 9. D, Grade 4 infection. White-seeded Kentucky Wonder hybrid (No. 780) inoculated with rust race 7.



Types of infection, grades 5, 6, 8, and 10, produced by several races of rust on different varieties of beans. *A*, Grade 5 infection. Kentucky Wonder Wax (No. 765) inoculated with race 14. *B*, Grade 6 infection. California Small White (No. 643) inoculated with race 15. *C*, Grade 8 infection. White-seeded Kentucky Wonder hybrid (No. 780)  $\times$  Pinto, inoculated with race 11. *D*, Grade 10 infection. Pinto inoculated with rust race 2. Secondary rings occur in connection with many sori.





size. If the physiological activity of the leaf was much reduced by aging before the pustules were mature, they remained undeveloped even on highly susceptible plants.

Immediately following inoculation the plants were placed in infection chambers and kept there for 24 to 48 hours, when they were removed to a bench in the greenhouse. An incubation period of 24 hours was as good as or better than any longer time. The infection chambers were constructed on one of the regular greenhouse benches. The front and back of the bench were built to the height of 1.5 and 2 feet, respectively, and the compartment was covered with a glass sash hinged at the back. Wet sphagnum was placed at the bottom of the chamber in order to maintain a high humidity. The infection chamber was divided into several individual compartments, each large enough to accommodate 100 or more pots. Under favorable greenhouse conditions, infection was evident in from 5 to 6 days after inoculation. The sori began to open a few days later, and the records of the degree of pustule development were made about 14 days after inoculation. They were sometimes taken a day or two later if it was suspected that development of the rust was delayed because of low temperatures or for other causes.

#### INFECTION RATING

The size of the rust pustule at the end of 14 days was the criterion for fixing the degree of susceptibility and resistance. Harter, Andrus, and Zaumeyer (3) previously used a scale of 0 to 10, 0 denoting immunity and 10 the highest degree of susceptibility, with intermediate grades between these two extremes. It is proposed to employ the same scale of graduation in this paper. Other investigators have used the same system, while some have used a scale of 0 to 100.

#### DESCRIPTION OF GRADES AND TYPES OF INFECTION

*Grade 0.*—Totally immune (pl. 1, *A*), no lesions or other evidence of infection.

*Grade 1.*—Necrotic flecks without spores (pl. 1, *B* and *C*). There is considerable variation in the general characteristics of the flecks on several of the differential varieties, caused by the various physiological races of the organism; some are very small and round, somewhat resembling the point of a needle (pl. 1, *B*), while others are angular in shape and vary greatly in size (pl. 1, *C* and *D*).

*Grade 2.*—This grade (pl. 1, *D*, and pl. 2, *A*) differs from grade 1 largely in that, although the sori are small, some spores are produced. The infection centers may or may not be surrounded by a necrotic area. Plants falling in this class are highly resistant.

*Grades 3 to 10.*—These grades (pl. 2, *B*, to pl. 3, *D*) are differentiated on the basis of the size of the spore-bearing pustules. Grade 3 (pl. 2, *B*, *C*), grade 4 (pl. 2, *D*), grade 5 (pl. 3, *A*), and to a less extent grade 6 (pl. 3, *B*) are regarded as commercially resistant, and higher grades up to grade 8 (pl. 3, *C*) are regarded as possessing some degree of tolerance.

Secondary and tertiary rings of sori (pl. 3, *D*) developed in many varieties of beans under certain conditions; however, their formation was not constant enough to be of any value in race identification. Rings were produced on some varieties more readily than on others, and it was by no means the more susceptible, but frequently the moderately susceptible ones, on which they appeared first. The

Bountiful, a variety that is not highly susceptible to any of the rust forms with the possible exception of race 10, frequently exhibited secondary rings in about 10 days and often earlier. There appears to be some correlation between the size of the secondary rings and resistance. It may be stated that, in general, secondary and tertiary rings were not produced in infections below grade 6. On the other hand, they were to be expected on all varieties with infections from grades 6 to 10.

Under greenhouse conditions susceptible and resistant plants inoculated with the same physiologic race often showed very different symptoms in the initial stage. Visible signs of infection appeared in about 5 days as small, nearly round and nearly white, immature pustules on the under side of the leaf beneath the epidermis. These juvenile sori gradually enlarged and in 7 to 10 days ruptured the epidermis. On the other hand, resistant plants of grade 1 and occasionally grade 2 usually showed brown necrotic lesions on the under side of the leaf in about 3 days. The lesions increased slightly in size and became a little darker in color, but there was little if any enlargement after 5 days. It was usually possible to determine resistant plants of grade 1 or 2 before there were any indications of infection in susceptible varieties.

## RESULTS

### IDENTIFICATION OF PHYSIOLOGIC RACES

Twenty different physiologic races of bean rust have been identified. Each one was separated from the others by its reaction on seven differential bean varieties. In only a few cases were any of the differential varieties entirely immune. There were, however, many examples of differentials being resistant, tolerant, or fully susceptible to different races. It was therefore frequently necessary to make use of degrees of infection of the differential varieties for the separation of the different races. In most cases degrees of infection were reasonably stable, although occasionally there were some departures from the standard, which sometimes rendered identification somewhat difficult. These variations in results occurred mostly with those varieties that showed some degree of resistance or were grown under unfavorable environmental conditions. For the most part they belonged to that group of varieties which the writers have termed commercially resistant.

A comparison of the reaction of a rust on the differential varieties with the data contained in table 1 should make identifications reasonably simple. A too close agreement must not always be expected, inasmuch as environmental conditions are known to influence the degree of infection, and mesothetic types sometimes confuse the results. Several tests may be required, but usually only one or two are necessary.

Reference to table 1 will show that U. S. No. 3 and No. 765 gave a "fractional" reaction to some of the races, that is, a difference in the degree of infection on the upper and lower sides of the leaf, the two readings being expressed in the form of a fraction. This behavior was not characteristic of any of the other differential varieties except No. 780 inoculated to race 19. U. S. No. 3 has shown an inconsistent tendency to fractional reactions with other physiologic races besides 6, 7, 8, and 20.

TABLE 1.—Reaction of the differential bean varieties to various physiologic races of bean rust (*Uromyces phaseoli typica*)<sup>1</sup>

Differential variety	Infection grade <sup>2</sup> produced by physiologic race -																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
U. S. No. 3	2	10	9	2	10	5-6	7	5	10	3	10	10	10	2	10	5	10	2	10	9
No. 181	8	8	8	8	8	3	3	3	9	9	8	8	9	8	8	8	7	7	8	8
No. 643	9	8	2	1	9	9	9	1	9	1	9	10	9	8	5	6	9	1	8	9
No. 650	10	10	10	10	10	10	10	10	10	2	10	10	10	10	10	10	10	10	10	10
No. 765	1	2	2	2	2	2	2	1	5-6	1	6	2	9	4-5	2	4	5	2	4	5
No. 780	1	2	1	1	2-2	2	4	2	2-3	1	6	3	10	9	2	9	1	5	4	9
No. 814	1-2	0	9	9	10	9	9	9	10	0	10	0	9	10	9	9	0	8	8	9

<sup>1</sup> These records represent the results obtained under the authors' criterion of the most favorable conditions. If environmental conditions are not favorable some variation must be expected, especially in the case of those varieties showing a high degree of resistance.

<sup>2</sup> Infection grades range from 0 for immunity to 10 for highest degree of susceptibility. The fractional expression of grade indicates different reactions on (numerator) upper and (denominator) lower surfaces of the leaf.

California Small White (No. 643) reacted peculiarly in several respects, and occasionally it was difficult to classify the several races as to the degree of infection. The lesions or pustules sometimes varied from grade 3 or 4 up to 8 to 10 on the same leaf when inoculated with a pure-line race. The infection was classed according to the grade that predominated on all the inoculated plants. This variety has consistently produced a small number of pustules in comparison with the other differential varieties.

Physiologic race 5 appears to be more prevalent and more widely distributed than most of the others. The investigations showed that there were a number of strains, closely related to but not identical with race 5, which did not differ enough from it to be classed as different races. The differences were mostly confined to the differential varieties Nos. 780 and 765. In table 1, race 5 is graded as 2 on No. 765 and 1 to 2 on No. 780; however, in the case of some strains of rust, a reading of 1 or perhaps 3 may occur on these two differential hosts. In other cases the grade may be below 2 on one of these differentials and a grade higher on the other. If there was no variation or only very little in the reaction of the differentials to the rust in duplicate tests, such differences might be sufficient to justify classifying the rust as a new race. In the absence of such constancy, the only other alternative seems to be to recognize the existence of subraces. In this connection it is proposed to create four subraces of race 5: Race 5-a includes those rusts that grade 0 on No. 780 and No. 765; 5-b, those that grade 1 on No. 780 and No. 765; 5-c, those that grade 1 on No. 765 and 0 on No. 780; and 5-d, those that grade 0 on No. 765 and 1 on No. 780, according to the scale adopted by the writers.

All the differentials except No. 181 showed considerable resistance to race 10. The Pinto (No. 650), a variety highly susceptible to all the other races and the one used as a standard with which reactions on all the differentials were compared, is quite resistant to race 10. It might appear that race 10 is a weak strain of rust; however, a large number of bean varieties are highly susceptible to it, as reference to table 2 will show. It was collected on the Bountiful variety, which was employed to keep the rust in an active condition in the greenhouse. While No. 780 and No. 765 are both resistant or at

least tolerant to most of the physiologic races, No. 780 is highly susceptible to races 13, 14, and 16 and No. 765 to race 13.

#### REACTION OF BEAN VARIETIES TO PHYSIOLOGIC RACES

The results of the relative susceptibility and resistance of a number of garden and field beans to one form of bean rust were published in 1935 (3). These varieties were graded on a scale of 0 to 10 and divided into three classes: Very susceptible, moderately susceptible, and slightly susceptible. The results showed considerable variation, some being highly susceptible while others were immune or highly resistant. In general the field beans were found to be more susceptible than the garden varieties.

In the present paper the varieties are graded on a scale of 0 to 10, according to the description given earlier for each grade. As new races were discovered they were inoculated to the different varieties; there now have been obtained ratings for resistance of most of the commercial varieties (table 2) to all rust forms except races 15 to 20.

TABLE 2.—Reaction of bean varieties to 14 physiologic races of bean rust

Group and variety	Infection grades <sup>1</sup> produced by physiologic race—													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
<b>Field beans:</b>														
Blue Pod	10	10	9	5	10	10	10						10	10
Boston Marrow	8	7	7	7	7	7	3			2				
Brown Swedish	10	8	8	8	7	6	4		7	10	8	7	5	
Great Northern	10	10	10	10	10	10	10	10	10	10	1	10	9	8
Kotenashi	10	10	9	10	10	9	10	8	10	1	10	8		
Large White Marrow	10	10	9	10	10	8	10	10	10	0	10	10		10
Marrow, Pea	10	10	10	10	9	9	7	10	10	9	10	10	10	9
Michelite	8	10	9	10	10	8	10	9	3	8	9	8	6	
Otenashi	10	10	9	10	10	9	10	10	10	10	10	9	8	10
Perry Marrow	8	5	7	7	7	7	6	3	10	1	6	10	7	8
Pink	9	10	10	10	10	10	10	10	10	1.5				
Red Kidney	10	10	10	10	10	10	10	10	10	2	10	10	10	10
Red Kidney, California strain	8	6	7	5	10	8	5	7	7	10	8	5	8	8
Red Kidney, Geneva	7	7	7	7	6	1	6		7	1	8	3.5	5	8
Red Kidney, Wells	8	8	7	7	7	6	6							
Red Mexican	10	10	10	10	10	10	10			1		10	9	10
Robust	9	9	2	10	10	10	10	10	10	2	10	10	10	10
Topary	10	10	10	9	9	9	9	4	7	2.5	9	9		10
White Kidney	3.8	5.8	7	8	8	8	7	4	6	3	5	3.5	6	
White Pea-bean, Geneva	10	10	9	10	10	10								
Yellow Eye, Improved	8		1.6					5	7	10	6	5		1
<b>Garden beans (pole):</b>														
Blue Lake (White Creaseback)	8	10	10	10	10	10	10	10	10	10	10	10	8	9
Casoknife, Early June	10	10	10	10	10	10	10	10	10	0	10	10	10	10
Casoknife, Dutch	6.9	10	9	2	10	7/4	2	8	10	0	10	10	2	5/2, 9
Cutshort (Corn Hill)	10	10	10	10	10	10	9	10	10	2	10	10	10	10
Everbearing	6	7	8	7	8	1	2							
Golden Cluster Wax	4.7	9	10	10	10	10	2	10	10	9	10	10	10	10
Horticultural, King Mammoth	2	9	7	2	7	1.3		0	1	8	4/2	0		0
Horticultural, London	1	9	8	0	7/3	1	0.3	0	1	8	1	0	0	0
Ideal Market (Black Valentine pole)	8	10	9	10	10	9	10	10	10	8		10	9	9
Ivory Stringless	1.0	8	8	0	7/3	1	1.4					6		0
Kentucky Wonder (Burker Stringless)	10	10	10	10	10	10	9	10	10	2	10	9		10
Kentucky Wonder (brown-seeded)	10	10	10	10	10	10	10	10		5			8	10
Kentucky Wonder (Morse 191)		10		2	10		10					10	4/2	4/2
Kentucky Wonder, Rust Resistant	2	1					9	6	9	1	10	9		9
Kentucky Wonder, U. S. No. 4	1	10	10	2	10	8/3	3	6	10	2	10	7		10
Kentucky Wonder Wax	2.6	4.8	9	2	9	9/3	1	2	9	0	10	4/2	4	5/2
Kentucky Wonder (white-seeded)	10	10	10	10	10	2	2	9	10	3	10	2	3	5/3
Lazy Wife (White Cranberry)	1	9	7	0	6/2	1	0	8	0	8	4/2	0	8	0
McCaslan	9	2	10	10	8	8	10	10	10	2	10	10	10	10
Missouri Wonder	10	2	10	10	10	10	2							

<sup>1</sup> Infection grades range from 0 for immunity to 10 for highest susceptibility. Two or more whole numbers indicate that different plants reacted differently. Fractions indicate different reactions on upper (numerator) and lower (denominator) surfaces of the leaf.

<sup>2</sup> *Phaseolus acutifolius latifolius* Freeman.

TABLE 2.—Reaction of bean varieties to 14 physiologic races of bean rust— Continued

Group and variety	Infection grades produced by physiologic race—													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Garden Beans (pole)—Continued.														
Oregon Giant		10	9	4	10	6/2	2	2	10	10	10	4/2	7	10
Phenomenon	2	10	10	4/2, 10	10	7/3, 10	2	5/2	10	1	19	10	1	10
St. Louis Perfection, White	10	10	10	10	10	10								
Striped Creaseback (Scotia)	10	10		10	10	10	10	10	10	10		10	10	10
Tennessee Wonder (Brown Sickle)	10	10	10	6	10	2	10	6	10	2	10	6/3, 8	10	10
Zeppelin pole	2	10	10	4/2	10	3	2		10	2	9	9		10
Garden beans (bush):														
Asgrow, Stringless Green Pod	9	9	8	9	8	2	2	4	8	10	8	6	1	1
Asgrow, White Seeded Wax	7	9	8	8	8	2	2		9	3	8	7	1	1
Black Seeded Green Pod	8	7	6	8	9		2		7	10	8	7	1	1
Bountiful	8	8	8	8	8	8	8	9	9	8	8	8-9	8	8
Brittle Wax (Round Pod Kidney Wax)	9	9	6	9	10		2, 6	6	10	10	8	8	2	1
Burpee Stringless Green Pod	4	9	8	6	5	5	4		7	10	8	8	6	4
Canadian Wonder	6	8	6	6	8	5	6	3	7	4	7	7	5	6
Challenger Black Wax	10	9	8	6	8	3	3		8	10	8	6	6	1
Commodore		8			10		1, 3	2, 4	8	10	7	8	1	
Currie Rust Proof Black Wax	3	8	7	7	6	2, 7	2, 4	2, 4	7	10	8	7	1	1
Davis Stringless Wax	7	8	8	7	7	6	6	6	5/7	10	8	7	7	6
Dwarf Black Wax	6	8	8	8	8	2	2, 5	4, 8	8	10	9	9	3	1
Dwarf French Master	9	9	8	8	7	7	4, 6		8	4	8	6	6	6
Early Mohawk	9	9	8	7	9	2	2	3, 6	8	10	8	8	1	
Flageolet Wax	6	6	6	7	2, 6, 6	1								
Flageolet White	1	8	8	1	8, 4	0	0			10		3	0	0
Fordhook Favorite	2	8	8	6	7	1	4	3	7	10	9	6	6	1
Full Measure	4	10	9	9	7	8	6	2	7	10	7	7		5
Giant Stringless Green Pod	3	9	9	9	8	9		8	8	10	9	8		8
Goddard (Boston Favorite)	8	7	8	8	10	9	8	7	8	10	8	9		8
Golden Crown	10	10	10	10	10	10	9							
Golden Eyed Wax	8	6	6	5	9		2	4	7	10		9	6	1
Golden Sword	1	5, 9	8	0	3	1	1	0	0, 3	10	5/3	1		
Grennell Rust Proof Wax	10	7	8	7	8	2	2	6	8	10	8	5	1	1
Hardy Wax	8	7	6	8	0		1	4	8	10	7	6	1	1
Hodson Wax	2, 4	9	10	10	9	2	2	7, 9	10	10	10	8		8
Horticultural, Dwarf	8	7	8	8	7	8	7	8	7	10	9	8	8	8
Horticultural, Dwarf (Cranberry)	0	7	8	0	6/3	0		6	8	10	8	9	8	
Horticultural, French	2	8	9	3	7	3	2	1	8	10	8	2		2
Improved Black Wax	7	9	8	8	7	2	3	3, 5	8	10	8	8	2	2
Improved Golden Wax	1, 3	9	8	8	8	2	8	6	8	9	8	7	1	1
Improved Kidney Wax	3	8	8	9	7	2	2	2, 5	8	10	8	5, 7		
Keeney Rustless Golden Wax	4	7	7	5	5	2	3	4	5	10	8		5	1
Konserva	9	10	7	8	7	7	7	8	7	10	8	6	9	8
Longfellow	5	9	8	9	6	7	6	5	8	10	8	6	7	9
Low Champion	2	7	8	8	6	0	2	3, 5	7	10	8	8	1	1
Masterpiece	10	10	10	10	9	9	10	3, 4, 8	9	1	7	7	7	8
Michigan White Wax	7	7	6	9	6	6	6	3, 6	7	10	9	7		8
New Sioux Stringless Wax	9	9	9	9	8	8	8	8	8	10		7		
Nonpareil	10	10	10	10	10	10	10	10	10	2	10	10	9	10
Penell Pod Black Wax	3	7	8	9	7	2	2	6	8	10		8		
Plentiful	5	7	5	7	10	5	5	5	5	9	5	8	6	7
Prolific Black Wax	8	7	8	8	7	2	2	4, 9	5	10	8	5	2	2
Refugee, 1000-1	2	5	6	6		8	2	6	8					
Refugee, Corbett	8	6	10	10	8	8	8		4	10	5	7	2	1
Refugee, Early	2, 7	8	7	8	7	1	2							
Refugee, Keeney Stringless														
Green pod	8	8	8	8	6	6	2, 4		7	8	6	6	2, 8	1
Refugee, Idaho	10	7	7	8	9	7	7		9	10	6	9	9	7
Refugee, U. S. No. 1	9	9	7	7	7	7								
Refugee, U. S. No. 5	7	7	7	7	7	7								
Refugee Wax	3, 7	8	7	8	6	2	1		4, 7	10	7	4, 6	2	1
Refugee, Wisconsin	6	4	8	8	8	1	2		8	8	5	8	1	1
Refugee, White-Seeded	8	6	4	3	8		1	6	4		6	6	2	1
Royal Purple Wax	6	8	6	6	7	3	4	5	5	8	5	5		
Rust Proof Golden Wax	2	8	7	7	8	2	2	5, 7	8	10	8	7	2	1
Stringless Kidney Wax	7	6	6	6	8	2	10		8	10		8		
Superba	8	9	8	9	8	2	3/2		3	2	6			1
Sure Crop Wax	3	8	8	8	8	2	0	5	8			7	2	1
Tendergreen	6	9	7	7	8	2	2	3	6	10	7	8	1	6
Tennessee Green Pod	10	10	10	10	10	10	10	8	10	0	10	8, 10	10	10
Unrivaled Wax	7	9	8	8	7	7	6	3		10	8	9	7	9
Valentine, Asgrow Black	6, 8	7	7	7	7	7	7	4	4	3	6	7	1	1
Valentine, Black	3	9	8	5, 8	7	1	3	4	4	10	4/7	3	6	1
Valentine, Red	4, 7	7	7	8	7	8	2	5	7	10	7	6, 8	1	6
Valentine, Red Stringless	8	8	7	8	7	6	7	6	6	10	8	5	0	7
Wardwell Kidney Wax	3	8	6	7	8	2	3	5	7	10	5	7		0
Weber Wax	1	9	8	9	9	2	3	4	9	10	7	7	1	2

Most of the seed for the variety tests was furnished by commercial seed growers, and in nearly all cases it was reasonably uniform in type and reactions, although considerable variation was occasionally found within some varieties, and this sometimes ranged from grade 0 to 10. The lack of uniformity occasionally confused the results, although in most cases the percentage of off-type plants was small. If the data indicated mixtures the grade of rust was determined from the reaction of the majority of the plants.

In general the field beans appear to be more susceptible to most of the physiologic races than the garden varieties. A striking exception is the case of race 10, to which the garden beans are more susceptible than the field varieties. Some of the garden varieties (Blue Lake, Early June Caseknife) are highly susceptible to most of the races, and some of the field beans (Geneva Red Kidney, White Kidney) are tolerant. In both the field and garden beans there are varieties (Oregon Giant, Great Northern, Brittle Wax, Hodson Wax) that are highly susceptible to some races of rust and resistant or tolerant to others.

Among the garden beans the pole varieties appear to be more susceptible than the bush types. The Kentucky Wonder types are naturally very susceptible and are often seriously damaged in regions where rust occurs. The Blue Lake, Early June Caseknife, and Cut-short varieties possess little or no tolerance to any of the rust forms, and there are several other varieties that are susceptible to all the races with one or two exceptions. On the other hand the King Mammoth Horticultural and London Horticultural, two pole varieties, are resistant or tolerant to more races than Dwarf Horticultural, a closely related variety of the bush type.

#### GEOGRAPHIC DISTRIBUTION OF PHYSIOLOGIC RACES

The geographic prevalence and distribution (table 3) of the different physiologic races in the United States were determined from rust material collected wherever found by the writers and from similar material sent them by pathologists and others from some of the agricultural colleges and experiment stations.

Previous to 1927, bean rust, although known to occur in many parts of the United States, was not as generally prevalent or destructive as it has been since. Sporadic outbreaks were reported by various investigators or observed by the writers in localities where very susceptible varieties were grown and the environmental conditions were favorable for rust infection; but epidemics such as some of those in recent years were exceptional, if they actually occurred.

After the rust had reached the epidemic stage over wide areas in several localities, a search was instituted for resistant varieties, and this eventually led to the discovery that not only one but possibly several different physiologic races occurred. The number of such races and their geographic distribution, as well as the reaction of different varieties to different races, were facts of much importance if attempts were to be made to develop resistant varieties by hybridization and selection. Preliminary inoculation tests with rusts collected from various parts of the country showed considerable diversity in results. Some varieties were highly resistant to a rust collection from a certain source but highly susceptible to one collected in a

different region. This information has been utilized in selecting material for the development of rust-resistant progenies.

The data shown in table 3 are too meager to be highly significant although some facts of interest are evident. The most outstanding lesson to be drawn from the results of the accumulated data is that there are no races specific for or representative of any one locality. The evidence shows that the races established in a locality in any one year may be followed by a different race or group of races the next year. In 1936 races 1 and 2 were identified from material obtained from Florida and some of the other States. In 1937 few collections were identified, but in 1938 race 1 was received only three times, and race 2 has been collected but once since 1936, and then from California. In 1938 races 5, 9, 10, and 11, and in 1939 race 5 were identified from Florida, but races 1 and 2 have not been received from that State since 1936. Races 1, 2, 3, and 4 were collected in Virginia in 1936; races 10, 14, and 16 in 1938; and race 10 in 1939. The few examples cited are typical of the entire country so far as physiologic races of bean rust are concerned.

TABLE 3.—*Distribution of the different physiologic races of bean rust collected in the United States and Hawaii, 1936-39*

State or Territory	Distribution of physiologic races of bean rust by years			
	1936	1937	1938	1939
Arkansas			5	
California			1	1, 2, 5, 10, 13
Colorado	1	4, 7, 8	1, 9, 13	
Florida	1, 2		5, 9, 10, 11	5
Hawaii			1, 12	
Louisiana			12	
Maine	4		5, 17	
Maryland	5		10	10, 20
Massachusetts	1, 2		5	
Michigan				5, 18
Nebraska			5	
New Jersey	4, 5		10	
New York			3, 10	
North Carolina			5	19
South Carolina	1, 2		15	
Virginia	1, 2, 3, 4, 6		10, 14, 16	10
Washington		1, 4		18
Wisconsin			5	

## DISCUSSION

The results of the investigations justify certain inescapable conclusions. It became apparent early in the prosecution of the problem and was more clearly demonstrated later that it was not always possible to obtain identical results from duplicate experiments. Differential host varieties often gave a slight difference in the degree of rust infection when inoculated with the same physiologic race at different times. In view of this fact it is essential that a too close agreement with previous results must not be expected. These variations were due to several causes. The greatest variations were obtained between inoculations made in the winter months and those made in the late spring and early fall, when the days were longer and the amount of sunshine much greater. Beans appear to thrive best with the maximum amount of sunshine and fairly high temperatures. During the winter months in the vicinity of Washington,



D. C., there are many cloudy days and frequently many consecutive days without sunshine. Under such conditions the plants lack vigor and are often icteroid. There seemed to be a direct correlation between plant vigor and the size of the sori. The sori on plants grown under reduced light were generally pale and smaller than those on plants grown with the optimum amount of sunshine. Temperature likewise influenced the size and general appearance of the sori and the tissue immediately surrounding them. If the plants were held in incubators or in an environment where the temperature was too high, the leaves became pale and the sori were undersized and slow to develop.

These inconsistencies and variations in the behavior of rust forms, attributed partly to environment, may possibly be explained in accordance with observations made by cereal rust pathologists who have proved the occurrence of mutations of various sorts in wheat rust. Newton and Johnson (5) selected a bright-orange and a grayish-brown mutant out of *Puccinia graminis tritici* (Pers.) Eriks. and Henn. Neither of the mutants showed any change in infection capability on the differential hosts. Both, however, differed markedly in viability and in the length of their urediospores. Johnston (4) secured from wheat in Texas a race of rust that differed in several ways from other known races. He found that its incubation period and time for full development were much longer than for other forms. The spores were lighter in color and smaller in size. He suspected, but offered no proof, that it may have originated as a mutation.

Interesting in this connection are the results of Newton and Johnson (5), who found mutations of wheat rust to be induced by the refrigeration of the urediospores. The mutation occurred in a form of rust that had been constant for 2 years. After the urediospores had been in storage for 6 months at a temperature of 8° C., inoculation experiments indicated a mixture of the original and a new form of rust. Four months later a new race had completely supplanted the old race and gave an entirely different reading on the differential hosts.

These facts may explain some of the difficulties the writers have experienced. It has been the practice to collect urediospores and store them in a refrigerator at temperatures below 0° C. for various lengths of time, or until they were needed. Viable spores have been kept in storage for more than 2 years, and markedly different results have been observed when they were used to inoculate plants at a later date. In no case, however, has a complete change been observed or has any new race been developed. The results, a priori, suggested a contamination but under conditions that made it difficult to explain how it came about. What happened to the race of wheat rust described by Newton and Johnson (5) may have occurred in bean rust that was stored by the writers at a considerably lower temperature.

More significant still are the results of Stakman, Levine, and Cotter (7), who demonstrated that not only did mutation take place in cereal rusts but that new races were isolated as the result of mixing the nectar of such species as *Puccinia graminis tritici* and *P. graminis agrostidis* Eriks. These results indicate that new physiologic races may originate from several causes.

The sori on the leaves of highly susceptible plants grown under optimum conditions were uniform in size and produced an abundance

of urediospores over a considerable length of time. On the other hand, not only may partially resistant or tolerant plants have a comparatively small number of sori but there may be several grades of infection, as for example grades 3, 7, 10, on the same leaf. So far as bean rust is concerned, a variety may react mesothetically to one or more physiologic races and not to others. California Small White (No. 643) frequently showed such a range in the grade of the sori that it was sometimes difficult to determine to which class they belonged. While it was highly susceptible to some physiologic races, there may have been many undeveloped sori that might fall in any grade from 3 to 10.

Differences in reaction among several stocks of a single variety have been the cause of considerable confusion. The Refugee is a good example. This variety is widely grown commercially. There are several different strains all of which doubtless originated from the same parental stock. However, the different seed companies have selected lines according to their own fancy and have obtained strains that react quite differently to a single physiologic race of rust.

How have new races of bean rust originated? The answer can be only a matter of speculation. Hybridization in fungi has been demonstrated, and the origin of new forms and races by mutation is generally accepted. The probabilities are that the different bean rust forms originated by one or the other of these methods, if not by both. Bean rust is autoecious and aecia have not been reported to occur under field conditions, but this circumstance does not necessarily preclude the possibility of their occurrence and their hybridization under natural conditions. On the other hand, no one has demonstrated that mutations have occurred in bean rust. It is probably safe to assume that the different physiologic races originated from a single ancestral line and that from it the new forms now known have developed either by hybridization or mutation. If these methods of origin are accepted, one is justified in concluding that new races will originate with increasing frequency in the future.

The distribution of the different physiologic races is shown in table 3. The collections are limited and a larger number from many more localities would be necessary before any general conclusions could be drawn respecting the distribution of the different races. Furthermore, it is believed that many more races could be discovered by more and wider collections. Foreign countries offer a fertile field from which to recruit new forms, and knowledge of such forms and the varieties of beans resistant to them is desirable in any comprehensive program for breeding rust-resistant varieties.

Bean rust epidemics are exceedingly sporadic except in a very few localities where they occur nearly every year. This indicates that rather exacting environmental conditions are probably required by the parasite.

Beans are grown in every section of the country either commercially or in the home garden, and in view of the facility with which the spores migrate their presence can be assumed. Yet epidemics are the exception rather than the rule, and there is no reason to assume that an outbreak will occur every year even though there is an abundance of spore material. Neither is there any reason to assume that if one race could start an epidemic all others could do the same thing under the same conditions, which would be equivalent to admitting that all

rices are identical in every way except in the choice of the bean varieties they infect. They are presumably individualistic. Temperature, humidity, and other environmental conditions that are optimum for one form of rust may not necessarily be optimum for another. If this is true, one form of rust might initiate an epidemic while a contemporary race under identical conditions could not. Several physiologic races are usually obtained in a single collection, but generally one greatly predominates in number of spores over the others. The form that causes the epidemic is probably the one best adapted to the conditions prevailing at the opportune time, when there is a timely compatibility between the host and the parasite.

In view of the large number of races of rust already known, what are the chances of developing resistant varieties? The difficulties increase with the increase in the number of specialized forms. A knowledge of the physiologic races themselves and the environmental factors that contribute to their behavior are prerequisites in any extensive program of breeding for rust resistance. The large number of commercial bean varieties on the market increases the difficulties of the problem. Most of these varieties hold a firm place in the trade and cannot be ignored. The susceptibility or resistance of most of the bean varieties has been determined for many though not all of the physiologic races. A careful study of table 2 (p. 724) will show that there are several varieties resistant to any one race of rust, and the proper selection of parents should ultimately yield productive rust-resistant progenies of the desired type. There are several varieties that are promising as parental material. Two of the differential hosts (Nos. 765 and 780) are resistant to many forms of rust. No. 765 is highly susceptible to only race 13, and No. 780 to only races 13, 14, and 16. Such varieties as the London Horticultural and Low's Champion also have promise.

The foreign field has not been thoroughly explored for resistant varieties and suitable parental stock. The material from foreign countries that has been examined by the writers was frequently composed of mixtures of many varieties and biotypes. Few of the varieties were pure lines and many were poor as to type and quality. Better progress is probably possible by making use of the varieties grown in the United States whose quality, type, and rust behavior are known.

#### SUMMARY

Twenty physiologic races of bean rust (*Uromyces phaseoli typica*) have been differentiated on the basis of their reaction to seven differential host varieties. The races were identified from bean material received or collected from many localities in the United States and from the Hawaiian Islands.

The grades of rust infection were arbitrarily established on a scale ranging from 0 to 10, immune varieties being graded 0, varieties with the highest degree of susceptibility, 10, and varieties with intermediate degrees of susceptibility being graded between these two extremes. Grades 3 to 5 or possibly 6 are regarded as commercially resistant. The degree of infection was found to be influenced by environmental conditions such as light, heat, and length of day. The sori were slow to develop and remained undersized when the inoculations were made during the cloudy and short days of the winter

months. A grade 1 infection in the winter months would often become a grade 2 or 3 on certain varieties if the inoculations were made in the spring or fall.

Necrotic lesions characteristic of resistance could be detected in about 3 days after inoculation on the under side of the leaf, while the flecks that develop later into large pustules were not evident before 5 days. Thus, evidence of resistance could be observed before there were any signs of infection on more susceptible hosts. The resistant necrotic lesions varied much in shape and size.

The susceptibility and resistance of a large number of commercial bean varieties were tested with races 1 to 14. A few varieties were highly resistant or immune to each race of rust, making it possible to obtain at least one parent variety that could be utilized in breeding for disease resistance.

Two or three physiologic races may be present in one locality during a single year. The number of spores of one usually greatly predominates over the others. These races do not necessarily recur in succeeding years in the same locality. They may be replaced by an entirely different race or races.

Urediospores can be kept in a viable condition for as long as 2 years if the leaves are dried for a few days at room temperature and thereafter stored at  $-20^{\circ}\text{C}$ .

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# RELATIONS OF NECTAR CONCENTRATION TO GROWTH OF ERWINIA AMYLOVORA AND FIRE BLIGHT INFECTION OF APPLE AND PEAR BLOSSOMS<sup>1</sup>

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## INTRODUCTION

The epidemiology of the blossom-blight phase of fire blight of apple (*Malus sylvestris* Mill.) and pear (*Pyrus communis* L.) incited by *Erwinia amylovora* (Burr.) Winslow et al. is very variable. The reasons for this variability are not satisfactorily understood. In recent years attention has been directed to nectar concentration as a factor influencing blossom infection.

Beutler (3)<sup>2</sup> studied the influence of various factors on nectar concentration, and showed that the concentration of nectar sugars stands in close inverse relationship to relative humidity. Thomas and Ark (10) showed that fire blight infection of apple and pear blossoms is favored by low concentrations and hindered or prevented by high concentrations of nectar. They proposed that the increase in volume and reduction in concentration of nectar during humid weather bears an important relation to the incidence of fire blight. Further evidence bearing on the relation of nectar concentration to fire blight infection was presented by Hildebrand (4), Hildebrand and Phillips (6), and Ark (1). These experiments relating to fire blight were conducted chiefly in the laboratory with cut blossoms of apple or pear or with cultures of *Erwinia amylovora* in comparatively large amounts of media in test tubes. The present paper reports further studies designed to aid in interpreting relations of nectar concentration to variability in the epidemiology of blossom blight. Special attention was given to work with trees in bloom, either in the orchard or under partly controlled conditions in the greenhouse. These studies were interrupted when one of the writers was called to another post. The available results, though in some respects fragmentary, are reported herein.

Experiments were directed along three major lines, as follows: (1) Growth and survival of the bacteria in various concentrations of nectar;<sup>3</sup> (2) measurements of nectar concentrations under natural conditions in relation to time, temperature, and relative humidity; and (3) relation of nectar concentration to the initiation of blossom infection.

<sup>1</sup> Received for publication February 5, 1940.

<sup>2</sup> Italic numbers in parentheses refer to Literature Cited, p. 742.

<sup>3</sup> Natural and artificial nectars were used. Armbruster (2) gives a general review of literature on nectar. Beutler (3) reports occurrence of invert sugars and sucrose in apple nectar in the proportions by weight of 12.8 to 8.5. Thomas and Ark (10) report analyses of pear nectar showing relatively less sucrose. The artificial nectar used in the present work contained invert sugars and sucrose in the proportions reported by Beutler (3) for apple nectar, 6.4 parts by weight of dextrose, 6.4 of levulose, and 8.5 of sucrose being dissolved in the following weak nutrient solution: Asparagine, 0.1 percent; sodium chloride, 0.01 percent; dibasic potassium phosphate, 0.05 percent; magnesium sulfate, 0.02 percent; calcium chloride, a trace. This nutrient-sugar solution, hereinafter referred to as artificial nectar or artificial nectar solution, was adjusted to pH 7.0.

## GROWTH AND SURVIVAL OF THE FIRE BLIGHT BACTERIA IN VARIOUS CONCENTRATIONS OF NECTAR

The influence of concentration of artificial nectar on the growth of fire blight bacteria in vitro was studied in Van Tieghem-cell cultures in which the amount of nectar and the number of bacteria approximated conditions encountered in nature. The cultures were prepared as follows: The nutrient solution in a large flask and the sugar mixture<sup>4</sup> in suitable amounts in test tubes were sterilized separately in the autoclave. Bacteria<sup>5</sup> from 1-day- to 2-day-old cultures were then introduced into the cooled flask in such numbers that a  $\frac{1}{400}$ -cc. drop of the complete medium would contain about 15 to 30 bacterial cells. Measured amounts of this nutrient solution were then added to the test tubes containing the sugars so that a series of seeded media was obtained containing, respectively, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, and 60 percent of sugars. In addition there were tubes that contained the weak nutrient solution with bacteria but without sugar, and others that contained the nutrient solution without bacteria or sugar. The concentration of the sugars in these media was checked by means of an Abbé refractometer and found to correspond to the desired percentage in each case. Hanging-drop cultures ( $\frac{1}{400}$  cc.) were made and placed in sterile Petri dishes. To prevent concentration of the solution through evaporation, the cover slips bearing the drops were sealed with vaseline all around the ring, except for a small opening to allow exchange of gases in the cell. For the same purpose a small amount of the solution (not seeded) from which the drop was taken was placed at the bottom of the cell and in the Petri dish. The Van Tieghem cells were prepared in triplicate for each nectar concentration and incubated for 6 days at about 20° C. The bacteria in the drops were counted at the end of the incubation period after fixing and staining.

The results showed that the bacteria multiplied considerably in drops containing no sugars and in those containing 1 to 10 percent of sugars, but they did not increase greatly in the drops containing 20 percent. No bacterial cells were seen in drops containing 30 percent or more of sugars, and no colonies appeared on nutrient-dextrose-agar plates when transfers from these drops were made. Growth increased sharply in the 1-percent drops and reached its optimum in the 2- to 4-percent drops, after which it diminished rapidly as the concentration of the sugars increased. The limiting sugar concentration for growth under the experimental conditions seemed to be between 20 and 30 percent. Some of the drops were kept for 14 more days, but no appreciable changes in the growth of the bacteria were noted. At this time the concentration of sugars in the drops was again measured with the refractometer and found to be about the same as at the beginning of the trials. The detailed data of this and two similar trials are given in table 1.

<sup>4</sup> See footnote 3, p. 733.

<sup>5</sup> Two isolates of *Erwinia amylovora* were used in these tests, one of medium pathogenicity and the other highly pathogenic. Both were progenies of single cells, isolated in hanging drops with microscopic control (11, 12).

TABLE 1.—*Growth of Erwinia amylovora in drops of artificial nectar of various concentrations in Van Tieghem cells*

Percentage concentration of sugars in nectar	Bacteria per cubic centimeter <sup>1</sup> in—			
	First trial	Second trial	Third trial	Average
	<i>Number</i>	<i>Number</i>	<i>Number</i>	<i>Number</i>
0	7,000,000	23,000,000	48,000,000	26,000,000
1	60,000,000	125,000,000		92,500,000
2	112,000,000	130,000,000	800,000,000	347,333,333
3	112,000,000	102,000,000		107,000,000
4	79,000,000	58,000,000	580,000,000	239,000,000
5	18,000,000	28,000,000		23,000,000
6	1,440,000	22,000,000	16,000,000	13,146,666
7	840,000	22,000,000		11,420,000
8	173,000	6,000,000	6,000,000	4,057,666
9	17,000	2,000,000		1,008,500
10		2,000,000	3,000,000	2,500,000
20	7,200	0	18,000	12,600
30		0	0	0
40				0
50		0		0

<sup>1</sup> Number is approximate. The isolate used in trials 1 and 2 was of medium pathogenicity; that used in trial 3 was highly pathogenic, faster growing, and produced more abundant growth on several media. Trial 1 was made in 1936, trials 2 and 3 were made simultaneously in 1937. In estimating the number of bacteria per cubic centimeter both dead and living organisms were included.

Tests were made on the survival of the fire blight bacteria in drops of concentrated artificial nectar in Van Tieghem cells. The size of drops and rate of seeding were the same as in the experiments just reported. Transfers from these seeded drops were made at successive time intervals to drops of artificial nectar containing 1 percent of sugars, a concentration that favors growth. The results showed that the bacteria survived in the nectar containing 20 percent of sugars for 48 hours, 30 percent for 24 hours, and 40 and 50 percent for less than 24 hours.

Pear blossoms were inoculated with bacteria that had stood for 24, 48, and 72 hours, respectively, in test tubes partly filled with artificial nectar solution containing 40 percent of sugars. The turbidity of the bacterial suspensions corresponded to No. 2 of McFarland's nephelometer (9). Enough bacteria survived for 72 hours in nectar containing 40 percent of sugars to induce infection. However, this experiment differed greatly from the trials in Van Tieghem cells in the amount of media used and the concentration of the bacteria.

#### RELATION OF AMOUNT OF INOCULUM TO INITIATION OF BLOSSOM INFECTION

Hildebrand (5) obtained no infection of blossoms of dwarf pear trees when approximately 100 individual cells were placed singly on the stigmas, anthers, and nectaries. Under similar conditions, however, infection resulted in 1 out of 2 trials on dwarf apple trees when a single cell was transferred to the nectary. The relative humidity during these experiments was about 50 percent. The concentration of the nectar was not measured. In another trial he obtained infection in 9 out of 15 cases when single cells were placed on apple nectaries of excised flowers held in moist chambers in a room at about 24° C. In



similar tests with increased numbers of bacteria 100 percent infection was obtained from all inoculations in which 10 or more cells were used.

Greenhouse infection experiments were made by the present writers on the blossoms of potted dwarf Bartlett pear trees when the concentration of the nectar sugars was kept at about 1 to 5 percent. Such nectar concentrations were induced by keeping the trees in a moist chamber for several hours or overnight, after which the receptacle cups were usually found to be filled or overflowing with dilute nectar. Bacteria from a highly pathogenic culture grown 36 hours on nutrient dextrose agar slants were introduced into the receptacle cups in  $\frac{1}{400}$ -cc. drops of sterile distilled water standardized to contain approximately 10, 100, or 1,000 bacterial cells. Care was taken not to wound the blossoms. From 32 to 48 blossoms were used in each trial. The results were as follows, the first figure in each group showing the approximate number of bacteria in the inoculum and the second the percentage of blossoms infected: 10, 66; 100, 91; 1,000, 93. These data, which are in general agreement with those of Hildebrand (5), indicate that 10 bacteria constitute a highly efficient inoculum when introduced into the nectar under sufficiently favorable conditions.

#### MEASUREMENTS OF NECTAR CONCENTRATIONS UNDER NATURAL CONDITIONS

Since nectar concentration may be a limiting factor in blossom-blight infection, it seemed desirable to study the concentrations actually encountered under natural conditions in Wisconsin orchards. Measurements were made by means of an Abbé refractometer, with record of time of reading, temperature, and relative humidity. The nectar was collected from one or more blossoms, depending on the quantity in which it was available. Illustrative results are shown in tables 2 and 3.

It is obviously not to be assumed that these limited data can be taken as representative or as covering the range of conditions that may be encountered in this State. The weather conditions, however, were not unusual. It may be noted that no rainfall occurred on the days of observation, except light showers on June 5 and 8, 1937 (table 3). In a large majority of the readings the nectar concentrations were well above those at which the fire blight bacteria can multiply.

The evidence of diurnal fluctuations in the nectar concentration of apple blossoms at Madison in the period of May 15-17 and at Sturgeon Bay, May 24-28 (table 2) is of especial interest in relation to potentialities for fire blight infection. The early morning reading in each case showed a concentration favorable for infection, whereas the readings taken later in the day usually did not. None of the readings on pear nectar at Sturgeon Bay in the period May 30-June 11 (table 3), however, showed nectar concentration favorable for infection, except the one made after the rain of June 8.

A striking aspect of these data, meager though they are, is the strong predominance of nectar concentrations that would preclude multiplication of the fire-blight bacteria and be disadvantageous for their survival.

TABLE 2.—Concentrations of pear and apple nectar under natural conditions, as related to time of day, temperature, and relative humidity, at Madison and Sturgeon Bay, Wis., 1936

Location and kind of blossom	Date in May	Hour of measurement	Temperature	Relative humidity	Approximate concentration of sugars in nectar
			°F.	Percent	Percent <sup>1</sup>
Madison:					
Pear	12	7:30 a. m.	65	87	2
Do	12	9:00 a. m.	71	69	
Do	12	2:30 p. m.	72	73	
Apple	12	2:30 p. m.	72	73	
Do	12	6:00 p. m.	67	68	
Pear	12	6:00 p. m.	67	68	
Do	13	9:00 a. m.	57	72	
Do	14	11:00 a. m.	63	61	
Crab apple	14	6:00 p. m.	63	60	48
Apple	15	7:00 a. m.	53	55	5
Crab apple	15	6:00 p. m.	73	50	48
Apple	16	9:00 a. m.	70	70	5-10
Do	16	12:00 m.	81	42	
Do	16	6:00 p. m.	83	40	
Do	17	11:00 a. m.	75	62	5
Do	17	4:00 p. m.	75	60	2-40
Sturgeon Bay:					
Apple	24	7:00 a. m.			5-10
Do	24	12 m.			2-21
Do	24	4:30 p. m.	73	65	2-70
Do	27	7:30 a. m.	60	79	1-10
Do	27	12:00 m.	71	53	
Do	27	6:00 p. m.	62	58	
Do	28	7:30 a. m.	51	72	4
Do	28	12:00 m.	70	55	

<sup>1</sup> Where no reading is given the receptacle cups were dry or nearly dry.<sup>2</sup> Measurements made on a small composite sample diluted with water, and percentage of sugars estimated.

TABLE 3.—Concentrations of pear nectar under natural conditions, as related to time of day, temperature, and relative humidity, at Sturgeon Bay, Wis., 1937

Date and hour of measurement	Temperature	Relative humidity	Concentration of sugars in nectar	Date and hour of measurement	Temperature	Relative humidity	Concentration of sugars in nectar
	°F.	Percent	Percent <sup>1</sup>		°F.	Percent	Percent <sup>1</sup>
May 30:				June 7:			
7:00 p. m.	70	68	36	7:00 a. m.	55	71	
May 31:				11:00 a. m.	64	56	
8:00 a. m.	82	69	36	8:00 p. m.	52	75	
12:00 m.	87	57	61	9:00 p. m.	52	58	
4:30 p. m.	86	57	59	June 8:			
8:00 p. m.	70	67	35	7:30 a. m.	54	88	(2)
June 1:				9:00 a. m.	55	76	3
8:00 a. m.	50	87	36	3:00 p. m.	60	58	
12:00 m.	59	63	50	6:00 p. m.	55	65	
12:45 p. m.	61	62	52	June 9:			
6:15 p. m.	58	83	38	7:45 a. m.	55	65	
June 3:				9:00 a. m.	60	49	
8:00 a. m.	62	70		2:15 p. m.	68	47	
6:00 p. m.	60	73		June 10:			
June 4:				11:30 a. m.	74	45	
10:30 a. m.	64	75		5:30 p. m.	69	64	
June 5:				June 11:			
6:30 a. m.			(2)	12:00 m.	83	46	
5:30 p. m.	60	97					
June 6:							
1:00 p. m.	66	53					
4:00 p. m.	59	68					

<sup>1</sup> Where no reading is given the receptacle cups were dry or nearly dry, unless otherwise noted.<sup>2</sup> Light shower. No reading made.<sup>3</sup> Wind.

## RELATION OF NECTAR CONCENTRATION TO BLOSSOM INFECTION

It was sought to check and supplement the evidence thus far discussed by blossom-infection experiments under partly controlled conditions in which the relations of nectar concentration were studied.

Field trials in which the blossoms were inoculated by means of a small camel's-hair inoculator with bacteria suspended in artificial nectar solutions of various concentrations were made at Madison and Sturgeon Bay in orchards in which no natural occurrence of fire blight was observed. The inoculated blossom clusters and controls were bagged during the incubation period. The results, which are given in tables 4 and 5, indicate that infection occurred freely when the bacteria were introduced in low concentrations of nectar, but that it was lacking or sparse when they were introduced in the highly concentrated artificial nectar. In this connection it should be noted that in at least one trial with concentrated nectar infection occurred long after the usual incubation period (footnote 3, table 4). It is thought that such infection may have occurred only after the nectar had become diluted through a natural process. It is likely that under field conditions the concentration of nectar may change considerably in a short time and thus facilitate, retard, or prevent infection after inoculation has taken place. This question was further investigated in experiments reported in the following paragraphs.

TABLE 4.—*Concentration of artificial nectar in which the bacteria were suspended in relation to blight infection of pear and apple blossoms, at Madison, Wis., 1936*

Kind of blossom	Concentration of sugars in the bacterial suspension	Treatment of blossoms <sup>1</sup>	Time inoculated and atmospheric conditions				Blossoms—	
			Date in May	Approximate hour	Temperature	Relative humidity	Treated	Diseased <sup>2</sup>
	Percent				<sup>a</sup> F.	Percent	Number	Percent
Pear	0	Inoculated without wounding.	12	7:30 a.m.	65	87	120	84
	0	Inoculated by puncturing.	12	7:30 a.m.	65	87	14	100
		Water drop without bacteria placed in receptacle cup.	12	7:30 a.m.	65	87	15	0
	1	Inoculated without wounding.	12	6:00 p.m.	67	68	30	50
	40	do	12	6:00 p.m.	67	68	18	<sup>3</sup> 0
	0	do	13	9:00 a.m.	57	72	30	50
	0	Inoculated by puncturing.	13	9:00 a.m.	57	72	10	100
	40	Inoculated without wounding.	13	9:00 a.m.	57	72	20	0
	40	Inoculated by puncturing.	13	9:00 a.m.	57	72	10	100
		Blossoms not treated, same branch. <sup>4</sup>	13	9:00 a.m.	57	72	50	0
	0	Inoculated without wounding.	14	11:00 a.m.	63	61	24	79
	1	do	14	4:00 p.m.	65	55	25	68

<sup>1</sup> Inoculations without wounding were made by placing a small drop of bacterial suspension in the receptacle cup by means of a small camel's-hair-brush inoculator.

<sup>2</sup> Records on disease development were taken about a week after treatment.

<sup>3</sup> Most of the inoculated blossoms were found diseased at a later inspection. Infection may have started later, after conditions in the receptacle cup became more favorable.

<sup>4</sup> On the same branch as puncture inoculations of the same date.

TABLE 4.—*Concentration of artificial nectar in which the bacteria were suspended in relation to blight infection of pear and apple blossoms, at Madison, Wis., 1936—Continued*

Kind of blossom	Concentration of sugars in the bacterial suspension	Treatment of blossoms	Time inoculated and atmospheric conditions				Blossoms—	
			Date in May	Approximate hour	Temperature	Relative humidity	Treated	Diseased
Apple	Percent				° F.	Percent	Number	Percent
	1	Inoculated without wounding.	14	4:00 p.m.	65	55	50	82
	1	Inoculated by puncturing.	14	4:00 p.m.	65	55	25	100
		Blossoms not treated, same branch. <sup>1</sup>	14	4:00 p.m.	65	55	73	0
	1	Inoculated without wounding.	16	11:00 a.m.	77	47	25	76
	5	do	16	11:00 a.m.	77	47	80	77
	40	do	16	11:00 a.m.	77	47	24	0
	40	Inoculated by puncturing.	16	11:00 a.m.	77	47	15	100
		Blossoms not treated, same branch. <sup>1</sup>	16	11:00 a.m.	77	47	23	0
	0	Inoculated without wounding.	17	10:00 a.m.	77	65	25	24
	0	Inoculated by puncturing.	17	10:00 a.m.	77	65	27	100
	2	Inoculated without wounding.	17	10:30 a.m.	76	63	35	51
	4	do	17	10:30 a.m.	76	63	25	44
	6	do	17	11:00 a.m.	75	62	24	0
	8	do	17	11:00 a.m.	75	62	25	0
	20	do	17	11:30 a.m.	75	62	25	0
	40	do	17	11:30 a.m.	75	62	25	0
	40	Inoculated by puncturing.	17	12:00 m.	75	62	26	100
		Water drop without bacteria placed in receptacle cup.	17	12:00 m.	75	62	32	0

<sup>1</sup> On the same branch as puncture inoculations of the same date.TABLE 5.—*Concentration of artificial nectar in which the bacteria were suspended in relation to blight infection of apple blossoms, at Sturgeon Bay, Wis., 1936*

Percentage concentration of sugars in the bacterial suspension <sup>1</sup>	Treatment of blossoms <sup>2</sup>	Blossoms—		Bacterial exudate present
		Treated	Diseased	
		Number	Percent	
0	Inoculated without wounding	28	28	Yes.
0	Inoculated by puncturing	7	100	Yes.
2	Inoculated without wounding	28	78	Yes.
2	Inoculated by puncturing	8	87	Yes.
4	Inoculated without wounding	17	61	Yes.
6	do	24	70	Yes.
10	do	45	46	Yes.
20	do	33	30	No.
20	Inoculated by puncturing	8	100	No.
40	Inoculated without wounding	27	7	Yes. <sup>3</sup>
40	Inoculated by puncturing	6	83	No.
	Water drop without bacteria placed in receptacle cup.	12	0	
	Blossoms bagged but not treated	48	0	

<sup>1</sup> A 0.3-percent supplement of sodium ricinoleate was added to each artificial nectar solution for the purpose of decreasing the surface tension of the solution.<sup>2</sup> See footnote 1, table 4.<sup>3</sup> The time of infection of these blossoms questionable.

In a greenhouse trial the blossoms of potted pear trees were inoculated with bacteria suspended in artificial nectar ranging in sugar concentration from 1 to 40 percent. Some of the trees received a 12- to 16-hour treatment in a moist chamber (?) at 20° to 22° C. immediately after inoculation, while others were left in the greenhouse without such treatment. One hundred percent infection occurred in blossoms inoculated with artificial nectar suspension containing 1 percent of sugars, whether the trees were given the moist treatment or not. Like results were obtained with blossoms inoculated with a nectar suspension containing 10 percent of sugars, with a moist treatment following inoculation. Considerable reduction in infection occurred in blossoms inoculated with this same nectar suspension with no treatment following inoculation, and in blossoms inoculated with a nectar suspension containing 40 percent of sugars with a moist treatment following inoculation. No infection occurred in blossoms inoculated with the suspension containing 40 percent of sugars but given no moist treatment after inoculation. In these trials no records were taken of the concentration of nectar in the blossoms during the incubation period. Such records seem necessary in order better to interpret some of the results obtained. The trials reported in the following paragraph afford such data.

Three successive trials were made in the field at intervals of several days, in which pear blossoms of an unknown variety were inoculated with fire blight bacteria suspended in water and in artificial nectar solutions containing 10 and 40 percent of sugars, respectively (table 6). On 3 successive mornings following inoculation, records were taken of the concentration of the mixed artificial and natural nectar and of the natural nectar of untreated blossoms. In trials 1 and 2 infection occurred only in blossoms inoculated with bacteria suspended in water. The sugar concentration of the mixed artificial and natural nectar and also of the natural nectar for the first 3 days after inoculation stood at 52 percent or higher. In some cases the nectar was so concentrated that no measurable samples were obtainable. During these 3 days the relative humidity was above 70 percent and no rain or heavy dew occurred. In the third trial, however, 100 percent infection occurred at the end of the incubation period in all the inoculated blossoms. The concentration of the mixed artificial and natural nectar and the natural nectar during the first 2 days following inoculation was relatively low, however, varying from 3 to 15 percent. During the 2 days light showers fell at intervals, the skies were clouded, the temperature was relatively low, and the relative humidity was near the saturation point for several hours at a time. Seven other similar trials were made during the same season at Sturgeon Bay with similar results.

TABLE 6.—Relation of nectar concentration to blight infection of pear blossoms in the orchard, at Madison, Wis., 1937

Trial No.	Concentration of sugars in artificial nectar in which the inoculum was suspended	Concentration of sugars in the natural nectar of controls at indicated time after inoculation			Concentration of sugars in the mixed artificial and natural nectar at indicated time after inoculation			Blossoms—	
		1 day	2 days	3 days	1 day	2 days	3 days	Inoculated	Diseased
	Percent	Percent	Percent	Percent	Percent	Percent	Percent	Number	Percent
1	0	(1)	(2)	(2)	(1)	(2)	(2)	48	62
	10	(1)	(2)	(2)	(1)	(2)	(2)	45	0
	40	(1)	(2)	(2)	52	56	68	92	0
2	10	(2)	(2)	(2)	(2)	(2)	(2)	60	63
	40	(2)	(2)	(2)	(2)	(2)	(2)	45	0
	0	3	15	(2)	62	60	66	68	0
3	10	3	15	(2)	2	11	(2)	65	100
	40	3	15	(2)	6	13	(1)	64	100
					12	14	42	120	100

<sup>1</sup> Sugar concentration not measurable.<sup>2</sup> Cups dry.<sup>3</sup> Rain fell a few hours after inoculation. Humidity continued high (90 to 99 percent) throughout the following day.

## DISCUSSION

When the bacteria were placed in small droplets of artificial nectar in Van Tieghem cells in the method used in the present work, they tended to be limited in growth at somewhat lower sugar concentrations and to survive for shorter periods than in the test-tube experiments reported in earlier work (1, 6, 10) or herein. Growth of the bacteria in artificial nectar and infection of unwounded blossoms inoculated in the nectar occurred freely under the conditions of these experiments only when the sugar concentrations were in the lower range encountered in natural nectar. Sharp inhibition or prevention of growth and infection occurred at the lower intermediate concentrations. There was usually no growth or infection at the medium and higher concentrations, which predominated in the orchard observations.

The data thus far available shed considerable light on the relations of sugar concentration to the growth of *Erwinia amylovora* in vitro and to blossom-blight infection following artificial inoculation. They show beyond doubt that nectar concentration can limit blossom-blight infection, but the detailed manner and extent of its influence on epidemiology seem still to be obscure in many respects. Are the results obtained in a limited number of experiments under artificial conditions a sufficient guide to the interpretation of what happens in the wide ranges of nature? Do we know what to expect when the bacteria are introduced into the nectar by insects? Will they be transmitted freely by nectar-sipping insects from one blossom to another when the nectar concentration is high? If so, will they survive and initiate infection when the nectar becomes sufficiently dilute? For what time and through what range of natural conditions can the bacteria thus survive in the concentrated nectar and still be able to incite infection? Do such insect-deposited bacteria commonly lie in wait in the concentrated nectar of apple and pear blossoms, ready to initiate infection as soon as a favorable dilution permits? Or

are there sharp limitations on insect transmission and survival of the bacteria in nectar? If so, what is their nature? Adequately controlled experiments involving natural transmission of the bacteria seem necessary for the elucidation of such questions. A beginning of studies on the experimental transmission of fire blight by bees and its relation to nectar concentration of apple and pear blossoms is reported elsewhere in this Journal (8).

### SUMMARY

The optimal concentration of sugars for growth of the fire blight pathogen, *Erwinia amylovora*, in  $\frac{1}{400}$ -cc. drops of artificial nectar in Van Tieghem cells was 2-4 percent. Growth rapidly decreased with increased sugar concentration, none occurring at 30 percent. The bacteria survived for 48 hours in similar drops containing 20 percent of sugars, 24 hours in 30 percent, and less than 24 hours in 40 or 50 percent. When tubes of artificial nectar containing 40 percent of sugars were heavily seeded, the bacteria survived for 72 hours and incited infection when placed in pear blossoms.

Approximately 10 bacteria in a  $\frac{1}{400}$ -cc. water drop constituted an effective inoculum when placed in dilute nectar of pear blossoms.

Abbé refractometer readings are reported for nectar of pear (*Pyrus communis*), apple (*Malus sylvestris*), and crab apple (*Malus coronaria*) collected under various conditions in nature, with record of time, temperature, and relative humidity.

Infection of unwounded pear or apple blossoms inoculated by placing small droplets of bacterial suspension in the nectar occurred freely only when the sugar concentrations were in the lower range encountered in natural nectar. Sharp inhibition or prevention of infection occurred at the lower intermediate concentrations. There was usually no infection at the medium or higher concentrations, which predominated in the orchard readings. However, infection occurred if the concentrated nectar was sufficiently diluted soon enough after inoculation.

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# TRANSMISSION OF FIRE BLIGHT BY BEES AND ITS RELATION TO NECTAR CONCENTRATION OF APPLE AND PEAR BLOSSOMS<sup>1</sup>

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## INTRODUCTION

The pioneer investigations of Waite (17, 18, 19)<sup>3</sup> and later experiments and observations by many others have established beyond doubt that the honeybee and some other insects are capable of transmitting the blossom blight of apple (*Malus sylvestris* Mill.) and pear (*Pyrus communis* L.) incited by *Erwinia amylovora* (Burr.) Winslow et al. For many years after this discovery insects were generally thought to be the only important agents for transmission of blossom blight. Later investigations (e. g., 2, 3, 4, 7, 10, 16), however, have shown that meteoric water is an important factor in its spread and that under some conditions minute aerial strands of bacterial exudate may be disseminated by wind. The relative importance of insect and water transmission, which seems to vary greatly with conditions, is subject to considerable difference of opinion. Since blossom blight is one of the most important phases of the fire blight problem, a better understanding of its epidemiology is highly desirable.

Though it is generally accepted that bees can transmit blossom blight, comparatively little experimental work has been done on the details of this transmission or on the factors that favor or limit it. It has been the purpose of the present work to contribute to a re-examination of these aspects of the problem in the light of recent information, especially in their relation to nectar concentration. These studies, which were pursued in the spring of 1936 and 1937, were unavoidably interrupted when one of the authors was called to another post. The available results are reported herein. A companion study on nectar concentration in relation to the growth of *Erwinia amylovora* and infection of blossoms following artificial inoculation is reported elsewhere in this Journal (8).

Literature on the transmission of fire blight has been reviewed by Parker (11) and others. Consequently, only papers that seem especially pertinent to the present work are cited herein.

## DIRECT TRANSMISSION FROM CONTAMINATED TO UNCONTAMINATED BLOSSOMS

An objective of the following experiments was development of methods whereby transmission of fire blight from one blossom to another by honeybees (*Apis mellifera* L.) could be studied experimentally under adequate control. Some needs for specific information

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<sup>3</sup> Italic numbers in parentheses refer to Literature Cited, p. 752.

concerning such transmission in relation to the epidemiology of blossom blight have been discussed elsewhere (8).

Experiments were performed in three ways: (1) Individual marked bees of an uncontaminated nucleus hive in a large cloth cage in the greenhouse successively visited inoculated<sup>4</sup> and uncontaminated blossoms of potted trees; (2) bees of an uncontaminated nucleus hive in a large cloth cage in the greenhouse freely visited two potted trees, one with inoculated blossoms and the other with uncontaminated ones; and (3) an uncontaminated bee, handled in a specially designed wire cage, visited inoculated blossoms and then uncontaminated ones in the greenhouse or the orchard.

Evidence that the bees were not contaminated with the fire blight bacteria at the beginning of the experiments is based on the following precautions and tests. The bees of the nucleus hives were brought from Louisiana in early spring before they had opportunity to leave the hive. If the hive had been contaminated in the preceding year, the evidence is strongly against the possibility that the bacteria would have overwintered in it (6, 11, 12, 14, 15). When the hives had been placed in the cloth cages, the bees were allowed on 2 successive days to visit uncontaminated blossoms of potted apple and pear trees that had been held in a moist chamber to bring the nectar to a dilution favorable for infection. Each individual bee used in experiments with the small wire cage was allowed to work on a few uncontaminated blossoms before it was employed in the subsequent transmission experiments. In all these control experiments the bees were seen to introduce the glossa into the receptacle cup and remain in position long enough to indicate that they were sipping nectar. Throughout all the experiments on transmission, no indication was found that the bees were contaminated before they were permitted to visit the inoculated or diseased blossoms.

Some typical experiments on transmission are described below as illustrative of the methods and results.

About 50 blossoms of a 3-year-old dwarf Bartlett pear tree in the greenhouse were inoculated in the nectar by means of a small camel's hair inoculator at about 6 p. m. The tree had previously been held for a few hours in a moist chamber (9) with the curtains wet but spray not running, in order to bring the nectar to a concentration low enough to favor infection.<sup>5</sup> After inoculation it was kept overnight in another moist chamber. On the following morning measurements of nectar by means of an Abbé refractometer showed that the sugar concentration varied from 3 to 5 percent, whether the blossoms had been inoculated or not. Platings were made from inoculated and uninoculated blossoms at 3 time intervals that day. Fire blight bacteria, which subsequently caused typical infection in inoculation tests, were isolated in all trials from inoculated blossoms, but in no case from uninoculated ones. On the morning after inoculation, this tree, with a like one that had received similar moist treatment but no inoculation, was placed in a cloth cage. Each tree was protected by mosquito netting, so that

<sup>4</sup> Unless otherwise stated all inoculations were made on the preceding day by introducing a drop of about 1/400 cc. of bacterial suspension into the receptacle cup by means of a camel's hair inoculator, with care not to wound the host tissue.

<sup>5</sup> Unless otherwise stated all potted trees, before and after being used in the transmission experiments, were placed overnight in the moist chamber at about 20° C., with the curtains wet but the water not running. The inoculated and the uncontaminated trees were kept in separate chambers. At the end of a moist treatment, the nectar in the blossoms was usually abundant, and contained 3 to 5 percent of sugars. On keeping the trees in the greenhouse for 1 hour at about 24°, the concentration of nectar sugars usually rose to 10 or 12 percent.

individual blossom clusters could be exposed to visitation by bees or covered at will. An uncontaminated nucleus hive of bees was then placed in the cage. An inoculated cluster was exposed, with the aim of having one bee sip nectar from the blossoms.<sup>6</sup> Most of the bees flew about above the trees but only a few actually approached the exposed cluster. As soon as one alighted on an exposed blossom it was marked on the thorax with a droplet of specially prepared aniline dye and no other bees were permitted to touch this cluster. This bee was allowed to work on the inoculated blossoms until it had a good chance to become contaminated but not long enough to get its fill of nectar. The inoculated cluster was then covered and one on the uninoculated tree was exposed. The marked bee, without returning to the hive, alighted on the blossoms and worked on each of them, at times returning to a blossom it had already sipped from before going to another it had not yet visited. Each blossom the bee touched was marked, and the order of visitation was recorded. After the bee had visited all the blossoms, it was caught and the cluster was covered. The glossa was cut off with sterile instruments and its apical part dipped successively into the nectar of 10 blossoms of a third available tree having abundant nectar of a concentration favorable for infection. The glossa and the honey stomach were crushed and plated. The three trees were then incubated overnight in the moist chamber at about 20° C., with the curtains wet but the water shut off. On the following morning the blossoms of all the trees were found to contain abundant nectar. The trees were then further incubated in the greenhouse for 7 days at about 22° to 24°, after which the results were taken. The trees were kept under observation for another month.

The results of this experiment show that the bee transmitted the disease from the inoculated blossoms to 2 of the 4 uncontaminated blossoms it visited. No disease developed in the 10 blossoms that were touched with the bee's glossa. The glossa, however, yielded a few fire blight colonies that were subsequently shown by inoculation to be pathogenic. The honey stomach yielded a great number of micro-organisms, none of which resembled the fire blight pathogen. Most of the blossoms inoculated with the camel's hair inoculator showed macroscopic symptoms of blight within 4 days. Blossoms that were not inoculated did not show any disease. The experiment just described was performed four more times under similar conditions. Only one of these trials gave positive results.<sup>7</sup>

Transmission was also accomplished in two trials of another type, in which an inoculated and an uninoculated tree were kept together in a cloth cage for 5 hours, the bees of an uncontaminated hive freely visiting the blossoms of both trees. In the first trial 32 of the 61 blossoms of a dwarf Bartlett pear tree (uninoculated when placed in the cage) became diseased within a week after the bees' visitation, and in the second 46 of the 87 blossoms on a Seckel pear tree (likewise uninoculated when placed in the cage) blighted. The disease was therefore transmitted to 52 percent of the blossoms of these two trees.

<sup>6</sup> Bees may visit blossoms to collect pollen or reconnoiter without sipping nectar. In these experiments they were regarded as sipping nectar when they extended the glossa into the receptacle cup and remained in position for a distinctly longer period than would be required for reconnoitering.

<sup>7</sup> The amount and concentration of nectar changed rapidly after the trees were taken out of the moist chamber. For instance, in one case the concentration of nectar sugars rose from 3.5 percent to 11 percent in 50 minutes. In a few hours the volume of nectar was so diminished that measurable samples were not obtainable. These changes in the amount and concentration of nectar during the course of an experiment may have influenced the amount of infection (8), notwithstanding the fact that the moist treatment of the plants after visitation by the bees induced nectar concentrations favorable for infection.

Most of the tests on direct transmission of blossom blight by selected individual bees were made with the use of a small wire bee cage or trap (fig. 1). The chief advantage of this cage is that it permits keeping a particular bee with known history through several operations in association with any selected clusters as long as the bee remains alive and active. The cage, made of 16-mesh galvanized wire screen, is about 10 inches long and 4½ inches in width and height. It consists of two detachable halves connected with hinges on one side and with a hook on the opposite side. Two sliding doors, each placed near the outer end of one of the hinged sections, cut off small compartments (fig. 1, *a* and *c*). A bee is easily caught with this cage and confined in the central large compartment (fig. 1, *b*). Then

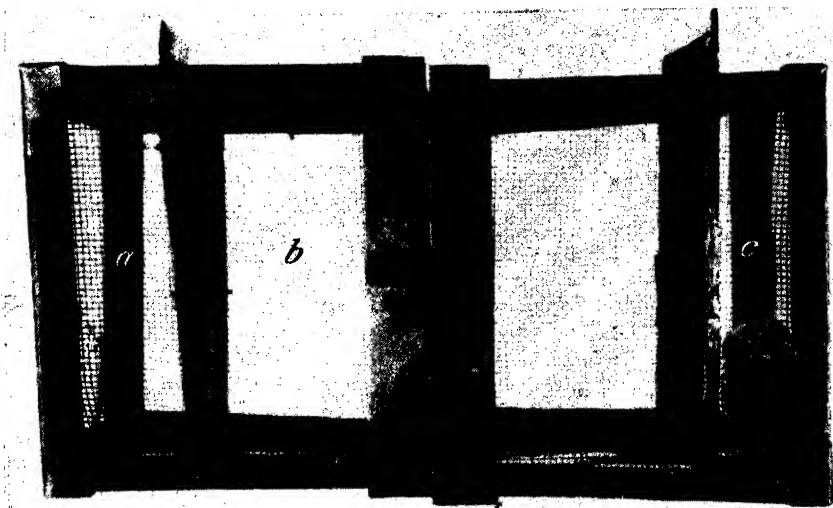


FIGURE 1.—Wire bee cage used in the inoculation experiments: *a* and *c*, Small outer compartments; *b*, large central compartment.

one of the trap doors is lifted and the insect is confined in the small outer compartment. Later the central compartment is opened, placed about a blossom cluster, then closed and hooked. The trap door is lifted, and the bee is allowed to visit the blossom cluster. After it has worked on the blossoms sufficiently, it is driven back into the small compartment and the sliding door is closed. The same bee is then used again on other blossom clusters, carried safely from one orchard to another, kept overnight, or handled otherwise according to the requirements of the experiment.

Transmission experiments with the wire bee cage included the following steps:

1. In an orchard in which no naturally occurring fire blight had been found, 10 blossom clusters of apple or pear were bagged separately, 2 of which were inoculated with the fire blight organism suspended in pure water or in artificial nectar<sup>8</sup> of various concentrations, as desired.

<sup>8</sup> The artificial nectar used contained invert sugars and sucrose in the proportions reported by Beutler (7) for apple nectar—6.4 parts by weight of dextrose, 6.4 of levulose, and 8.5 of sucrose being dissolved in the following weak nutrient solution: Asparagine, 0.1 percent; sodium chloride, 0.01 percent; dibasic potassium phosphate, 0.05 percent; magnesium sulfate, 0.02 percent; calcium chloride, a trace. The solution was adjusted to approximately pH 7.0. The details of preparation are reported elsewhere (8).

2. About 1 to 3 days later, a bee was caught in the sterilized cage and allowed to sip nectar from two of the uninoculated clusters. The total number of blossoms worked on was noted.

3. As soon as the cage could be shifted into position, the same bee was allowed to sip nectar from an inoculated cluster.

4. After a similar brief interval, the same bee was allowed to work on four of the uninoculated blossom clusters.

5. The bee then was decapitated, its glossa and honey stomach plated, and the pathogenicity of the recovered bacteria tested.

In some special trials the concentration of the nectar in the blossoms was measured just before or just after the bee's visit.

By using the technique just described, in some cases omitting steps 1 and 5, 32 transmission tests were made in the greenhouse or the orchard at Madison in 1936. Ten of these gave positive results; i. e., the bee transmitted the disease from an inoculated to an uncontaminated blossom.

The transmission tests were continued during the same season at Sturgeon Bay, Wis., where the blooming season is later than at Madison. In all cases the transmission was attempted with single bees, trapped in the wire cage. Of the 26 individual tests, 7 gave positive results, 13 negative, and 6 doubtful. Of the tests that gave positive results, 5 were made when the receptacle cups were moist or wetted and only 2 when they were apparently dry. None of the 13 tests that gave negative results were made when the receptacle cups were moist or wetted.

#### LENGTH OF TIME AFTER INOCULATION THAT BLOSSOMS ATTRACT BEES

One experiment was performed to gain evidence on the length of time after inoculation that blossoms will attract bees and serve as sources of contamination. It consisted in placing with an uncontaminated hive in the cloth cage two different Bartlett pear trees each day, one inoculated and the other not. The inoculated tree introduced on the first day had been inoculated 5 days; that on the second, 4; the third, 3; the fourth, 2. The results showed that under the conditions of the experiment the bees could transmit the disease to healthy blossoms from diseased blossoms that had been inoculated for 5 days. The diseased blossoms on the tree introduced 5 days after inoculation were already wilted and light brown in color. Three bees on more than 5 occasions touched these diseased blossoms with the glossa, then moved to the healthy blossoms. It was evident, however, that the healthy blossoms attracted more bees than the diseased ones and that the bees lingered longer on healthy than on diseased blossoms. The tree that was uninoculated when placed in the cage with the tree inoculated for 5 days had 46 blossoms, 29 of which were found diseased 10 days after the bees' visit.

#### CONCENTRATION OF NECTAR IN BLOSSOMS AT THE TIME OF THE BEE'S VISIT IN RELATION TO TRANSMISSION

In a greenhouse trial (table 1) some potted Bartlett pear trees were given a treatment in the moist chamber that brought the sugar concentration of their nectar within a range of 3 to 8 percent. They were then placed in a cloth cage and subjected to visitation by contaminated bees with similar trees that, having received no moist treatment, had

nectar with a sugar concentration of 45 percent. After removal from the cage the trees with low nectar concentration received a second moist treatment, whereas the others did not. Thirty-eight percent of the visited blossoms with the lower nectar concentration and none of those with the higher blighted.

TABLE 1.—*Nectar concentration in blossoms of 2-year-old Bartlett pear trees in relation to transmission of fire blight by contaminated honeybees*<sup>1</sup>

Bee No.	Moist treatment of blossoms before or after bee's visit <sup>2</sup>	Concentration of sugars in nectar before bee's visit <sup>3</sup>	Blossoms visited by bee	Blossoms diseased	
				Number	Percent
1	Treated before and after	3, 4, 8	3	2	3
1	do	3, 4, 8	5	2	
2	do	3, 4, 8	3	0	
2	do	2, 3	4	1	
2	do	2, 3	2	2	
3	do	4, 5	4	1	
4	No moist treatment	45 or higher	4	0	
4	do	45 or higher	5	0	
4	do	45 or higher	5	0	
5	do	(1)	3	0	
6	do	(1)	4	0	

<sup>1</sup> The bees had just been contaminated by sipping nectar from blossoms artificially inoculated on the preceding day. The experiments were performed in the greenhouse at 20° to 24° C.

<sup>2</sup> A bee visited from 2 to 5 blossoms of a cluster.

<sup>3</sup> Values are for individual samples. The concentration of nectar rose in some of the blossoms to 15 percent during the time the bee worked and before the tree was put back into the moist chamber.

<sup>4</sup> Receptacle cups dry.

In a field trial it was aimed to control in part the nectar concentration of the blossoms from which the bees obtained inoculum, as well as of those to which they were to carry it. In a pear orchard in which no naturally occurring blight had been found, blossoms were inoculated on various days with fire blight bacteria suspended in artificial nectar solutions with sugar concentrations varying from a trace to 40 percent. At the same time small drops of artificial nectar of the same range of concentration, but containing no bacteria, were placed in the receptacle cups of uncontaminated blossoms. Some of the treated blossoms were bagged in an attempt to check the rapid increase of nectar concentration. On the following day individual uncontaminated bees were allowed to sip nectar, first from some of the inoculated blossoms, then from uninoculated ones containing the artificial nectar drops. Shortly before or after the bees visited the blossoms, the concentration of the nectar in these and other blossoms was measured. It was found in most cases that the concentration had undergone changes. Some of the drops that originally had contained a trace or 1 percent of nectar sugars were later found to contain as high as 10 or 12 percent. Likewise, blossoms that originally contained 40 percent of nectar sugars were found to have 70 or 75 percent. The results of these trials, which are summarized in table 2, show that when a bee worked on inoculated blossoms with nectar containing 2 to 12 percent sugars and then on uncontaminated ones with nectar containing 0 to 35 percent sugars, 49 percent of the latter group blighted. A higher percentage of infection might have resulted if the concentration of nectar in some of the blossoms had not risen so high. On the other hand, no infection resulted when the bees first worked on blossoms with nectar containing, respectively, 10-14, 42-56, and 48-75 percent sugars and then on others with nectar containing, respectively, 10-18, 44-47, and 46-70 percent. Likewise, bees that

worked on inoculated blossoms with apparently dry receptacle cups and then on uncontaminated ones with dry receptacle cups did not transmit the disease.

TABLE 2.—*Concentration of pear nectar in relation to transmission of blossom blight by honeybees, Sturgeon Bay, Wis., 1937*<sup>1</sup>

Range of concentration of sugars in nectar of inoculated blossoms from which bees sipped	Range of concentration of sugars in nectar of healthy blossoms from which contaminated bees sipped	Bagging of blossoms after bees' visit	Bees used	Blossoms	
				Used	Diseased
Percent	Percent		Number	Number	Percent
2-8	0-10	Bagged	12	50	56
3-12	0-35	Not bagged	8	42	40
10-14	10-18	Bagged	6	22	0
42-56	44-47	do	10	34	0
48-75	46-70	Not bagged	9	36	0
(2)	(2)	do	18	52	0

<sup>1</sup> Wire cages were used for controlling the bees and the experiments were performed on orchard trees.

<sup>2</sup> Receptacle cups dry.

## DISCUSSION

It is recognized that some degree of artificiality may attend all experiments with bees handled in captivity, and that results from such work are reliable only in proportion to the adequacy with which they are observed and controlled.

Transmission of the disease from one blossom to another was demonstrated by each of the three methods tried, and each method may be useful. However, work with individual bees greatly facilitates adequate observations and controls. Use of the bee cage substantially increases the range and flexibility of experimentation with individual bees.

The large number of instances in which blossoms did not blight after visitation by a contaminated bee indicates that there are important limitations on the efficiency of this insect in transmitting the disease. Indeed, if this were not the case, it would be very difficult to understand how our apple and pear culture could continue, in view of the great number and activity of bees.

The results of these experiments on transmission of blossom blight by bees indicate that nectar concentration is a very important factor limiting this mode of transmission. They are in general accord with the results of studies (5, 8, 13, 14) of nectar concentration in relation to fire blight infection initiated by artificial inoculation. However, in many cases in which the nectar was at a favorable concentration, little or no infection occurred after contaminated bees had sipped from it. It is, therefore, apparent that other factors besides nectar concentration are important in limiting blossom-blight transmission by bees. An experimental study of such factors lies beyond the scope of the present paper.

The need for information on factors favoring or limiting blossom-blight infection under conditions of natural transmission has been discussed elsewhere (8). The present investigation was interrupted soon after experimental methods for such work had been developed. Further studies under various conditions are needed. It would seem especially desirable to perform additional greenhouse and orchard



experiments in which contaminated bees visit uncontaminated blossoms containing nectar too concentrated to permit infection. The time during which bacteria thus deposited will live and the range of conditions they will tolerate without losing the capability to infect when favorable conditions occur are vitally important considerations in relation to the epidemiology and control of the disease. While work with artificial inoculation is very valuable in helping to define and interpret problems relating to blossom-blight transmission by bees and other insects, further experimental work on transmission by the insects themselves seems essential to an adequate understanding of their role in disseminating the disease.

### SUMMARY

Transmission by honeybees of fire blight of apple (*Malus sylvestris*) and pear (*Pyrus communis*), incited by *Erwinia amylovora*, was studied by three experimental methods. The most flexible and convenient one employed individual bees handled in a specially designed wire cage.

Bees were attracted to blighting blossoms that had been inoculated 5 days before, and transmitted the disease to healthy blossoms.

In greenhouse and orchard experiments contaminated bees freely transmitted blight to healthy blossoms when the sugar concentration of the nectar was in the lower range encountered in nature, but not when it was in the medium or higher range.

In the experiments reported herein, nectar concentration was an important factor in limiting blossom-blight transmission by bees. However, in many cases in which the nectar was at a favorable concentration, little or no infection occurred after contaminated bees had sipped from it. It is apparent that other factors in addition to nectar concentration are important in limiting blossom-blight transmission by bees.

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